COMPARATIVE SEROLOGY OF SOME BRACHYURAN CRUSTACEA AND STUDIES IN HEMOCYANIN CORRESPONDENCE [1]

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Introduction

Researches in systematics may be conducted by comparing the serum or corresponding body proteins of organisms using serological methods. The underlying principle of serological systematics is that the proteins compared are representative of the organisms producing them in the same sense that their corresponding structures are and for the same general reasons. Of the serological reactions used for such researches the precipitin reaction has undoubted advantages. An extension of the previous precipitin studies in the serological systematics of Crustacea and related problems is the content of this report.

General Materials and Methods

Antigens

The sera of the Crustacea compared in this study were in part provided by Dr. Alan A. Boyden [1] who gathered them during summers over a period of years at various biological stations along the Eastern coast of the United States and elsewhere, namely, Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, 1936; U. S. Bureau of Fisheries Laboratory, Beaufort, North Carolina, 1936; Tortugas Laboratory, Carnegie Institution, Washington; Key West, Florida, 1932, 1934, 1936, 1939; also at the Marine Laboratory, Citadel Hill, Plymouth, England, 1939, 1948.

In addition to the samples of sera, the author had access to Dr. Boyden's original records made at the time of collection, plus the correpondence incidental to the collection and identification of particular samples. These records were of great help in orientation to the work and in providing useful hints and suggestions which saved countless hours when the author did his own field collecting.

Useful serum samples were secured by the writer in the field at Barnegat Bay and Delaware Bay, New Jersey. Samples of European crustacean sera were collected in the Summer of 1948 by the author at the following European biological laboratories: Museum National D'Histoire Naturelle Laboratoire Maritime, Dinard, France; Universite de Paris, Biologie Marine, Laboratoire Arago, Banyuls-sur-Mer, France; and the Stazione Zoologica, Napoli, Italia.

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In general the blood was taken from the crabs by removing the 5th pereiopod and permitting the blood of the organisms to drain into porcelain pans or glass crystallizing dishes. The crabs were held over the collecting pans by means of an ordinary clamp attached to a cross bar supported between two vertical stands. In the case of the long-tailed Crustacea such as crayfish and lobsters, the blood was collected by slitting the abdomen ventrally where it joins the thorax and holding the organism by hand over a large collecting funnel making sure the posterior end of the abdomen was pressed firmly to the lip of the funnel as an insurance against sudden abdominal flexures. It is necessary to occasionally break the jelly-like clots that form at the openings from which the blood is draining in order to obtain good yields from each animal. In those cases where the organisms were small the blood was taken from convenient points within the body cavity by means of needle and syringe. Piercing the body at the base of the appendages usually gave good yields. It is interesting to note here that placing the needle in the heart or in the pericardium gave poorer results than when a sinus some distance from the heart was tapped.

TABLE I

List of the species of Crustacea used as antigens

Acanthocarpus alexandri Stimpson
Callinectes marginatus (Milne-Edwards)
Callinectes sapidus Rathbun
Cancer anthonyi Rathbun
Cancer borealis Stimpson
Cancer irroratus Say
Cancer magister Dana
Cancer pagurus L.
Carcinus maenas L.
Geryon quinquedens Smith
Maia squinado Rondelet (Herbst)
Menippe mercenaria (Say)
Mithrax verrucosus Milne-Edwards
Ocypode albicans Bosc.
Panopeus herbstii Milne-Edwards
Panulirus argus (Latreille)

The collected bloods were permitted to clot and the sera to be expressed. Centrifugation at 2000–3000 r.p.m. for 20 minutes was usually employed to clear the sera. It is feasible to hasten the expression of serum by wrapping the clot in a double thickness of clean, fine-mesh, bolting or cheese cloth and twisting the wrapping by hand. The sera were sterilized by Seitz filtration, bottled in serum vials and stored at $3° \pm 1°$ C.

Table I is a list of the Crustacea whose sera were used as antigens in this work. The identifications of North American Crustacea were made with the works of Pratt (1936), Faxon (1898), and the remarkable publications of Rathbun (1917, 1925, 1930, 1937). The French fauna was checked against Bouvier (1940). The fauna of other European countries was identified in the works of Borradaile (1907), Pesta (1918), and Thiele (1935).

Antisera

All antisera were produced in rabbits. Intravenous and/or subcutaneous routes of injection were employed in the production of antisera. Inasmuch as it is impossible to ascertain at present the degree to which a rabbit will respond to a given amount of antigen, and since this response is perhaps the greatest variable in present day serological studies, what was believed to be minimal amounts to produce a good response were used. Generally 0.25 cc. was given on the first injection followed on alternate days with three 0.5 cc. injections. In every instance good, usable antisera were obtained when the hemocyanins were injected in these doses. In some cases a presensitization technique was employed to improve the strength (i.e. precipitating capacity) of the antisera. These rabbits were given 0.25 cc. of antigen intravenously and then permitted to rest for 30 days. This was followed by a series of 4 subcutaneous injections given on alternate days.

TABLE II

List of antisera

Antiserum	Homologous antigen	Remarks
I–63	*Menippe mercenaria* 36-A	Prepared by Dr. Alan A. Boyden Rutgers University
I–76	*Panulirus argus* 39–1	
I–77	*Panulirus argus* 39–1	
I–78	*Cancer borealis* 3a1	
I–86	*Callinectes sapidus* 47–1	
I–87	*Callinectes sapidus* 36–1	
I–94	*Cancer borealis* 1a	
I–95	*Maia squinado* No. 5	
I–97	*Cancer pagurus* No. 3	
I–98	*Menippe mercenaria* 36–A	
I–99	*Ocypode albicans*	
I–100	*Geryon quinquedens* 36–1	
I–101	*Cancer irroratus* 36–1	
I–105	*Geryon quinquedens* 36–1	
I–106	*Cancer irroratus* 36–1	
I–107	*Acanthocarpus alexandri*	
I–110	*Geryon quinquedens* 39–1	
I–114	*Callinectes sapidus* 47–1	

Table II is a list of antisera prepared and utilized by the author except where specially annotated.

Bleeding the rabbits to secure the antisera was accomplished in either one of two ways. Small samples of blood were withdrawn from the central artery of the ear, by using a number 22 gauge needle and syringe. For complete bleedings blood was withdrawn directly from the heart by cardiac puncture. Size number 18 gauge needles and 50 cc. syringes were used in this latter procedure. All antisera were centrifuged, sterile filtered through Seitz filters, and stored in the refrigerator until used.

Method of testing

The Libby photronreflectometer (1938) was utilized exclusively in the measurement of turbidities developed as a result of the interaction of antigens and antibodies. The technique employed was essentially the same as that described by Boyden and DeFalco (1943). Minor variations in technique as developed by the writer were matters of convenience and did not represent any major changes in their method of making the dilution series (see Figs. 1 and 2) of the antigens, nor in the use of the machine. Two recent papers (Boyden et al., 1947; Bolton et al., 1948) have analyses of the performance of the photronreflectometer and report the conclusion that for white precipitate systems, which include all the precipitin reactions, the instrument is unsurpassed at present in its sensitivity and range of usefulness in studying the characteristics of precipitates.

For all tests the procedure in which the amount of antiserum is held constant and the amount of antigen is varied was employed. The reacting cells of the photronreflectometer have a 2 cc. operating level and this volume was used in all testing. Final volume for each antigen dilution was always 1.7 cc. to which 0.3 cc. of immune serum was added to make up the 2 cc. volume. Turbidities (i.e. galvanometer readings) inherent in the fluid of the antigen dilution, and those due to dirt or blemishes on the glass of the reacting cells plus the turbidity characteristic for 0.3 cc. of each antiserum used were deducted from the total turbidity developed in each reacting cell. For these reasons the resultant turbidities can be considered as those due to the interaction of antigen and antibody.

The range of antigen dilutions regularly employed was between 4000 and 1 gamma of protein per cubic centimeter or solution, or in terms of dilutions of protein from 1 : 250 in doubling series to 1 : 1,024,000. When necessary the range of antigen dilution was extended. For the sake of convenience in plotting the results of the reaction on graphs, the antigen dilution cells were assigned numbers in a chronological sequence with cell No. 1 containing the greatest concentration of antigen. Figure 1 shows graphically a typical dilution sequence.

For all of the work in this paper the precipitin turbidities were those developed during 20 minutes incubation in a dry-air incubator maintained at 38° C.

The titration curve

The graph of the turbidities developed for serological tests is usually made in this laboratory with the turbidities plotted as the ordinate and the antigen dilution as the abscissa with the greatest concentration of antigen nearest the x-y axes intersect. The turbidities rise to a maximum, then decline again to a minimum following generally a normal distribution curve. By assigning unit distances between the geometrically changing antigen concentration values along the abscissa, the curves as plotted are more or less symmetrical. Skewness toward the region of antigen excess is quite common. Variations in the amount of kurtosis have also been observed. The amounts and kinds of both antigens and antibodies in the solutions combine to provide many variations of this type of frequency distribution. Figure 2 represents an idealized titration curve. The numerical value used to characterize any one particular curve for comparative purposes is that obtained by summating the turbidities over the whole reaction range. This value is proportional

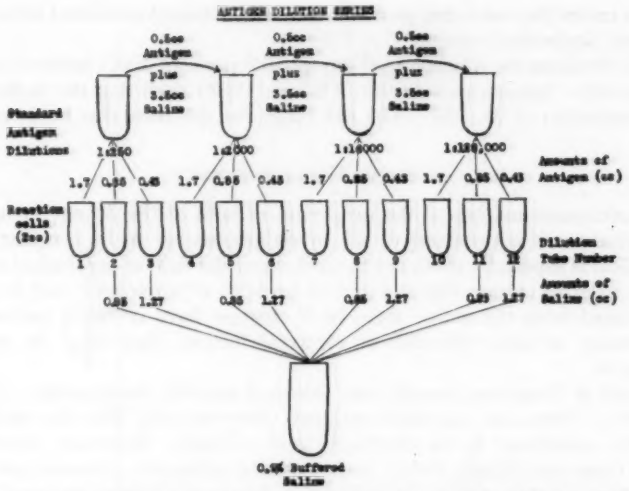

FIGURE. 1. A diagram of the technique used in preparing a typical doubling-dilution series of a given antigen for use in the Libby Photronreflectometer. The initial standard antigen dilution (1:250) is prepared directly from a serum of known protein concentration. The subsequent standard antigen dilution tubes are prepared from the first. Each reaction cell has a constant antigen dilution volume of 1.7 cc. to which is added and mixed a constant volume of 0.30 cc. of antiserum (α procedure).

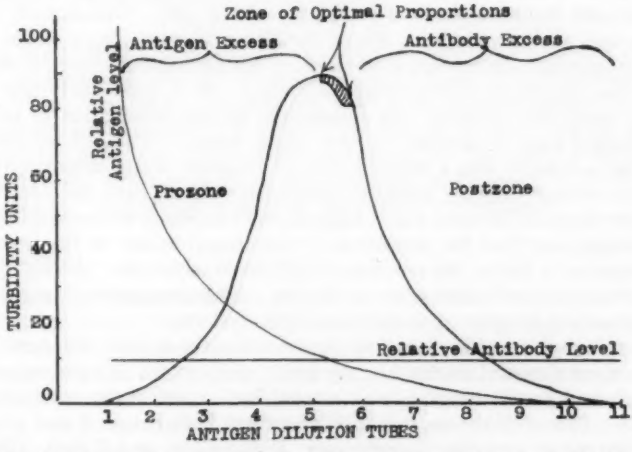

FIGURE 2. A diagrammatic representation of a typical titration curve obtained by using the Libby Photronreflectometer as the turbidimeter, and the technique of reacting varying antigen dilutions with a constant amount of antibody. Included also are appropriate curves to portray the relative antigen and antibody levels in each antigen dilution tube. For these latter curves the turbidity units of the ordinate axis do not apply.

to the area under the curve and provides an easily obtained statistical index of a very complicated biochemical system.

For all titrations the dilution medium was 0.9 per cent NaCl buffered with M/15 phosphate salts (Sorensen's solution) (Evans, 1922) such that the buffer was in a final concentration of M/150. The pH range for the tests was between 7.05 and 7.15.

Antigen Correspondence

The continuous analysis of the properties of both of the primary reagents used in serological work is necessary if the investigations are to be considered critical. This problem is especially pertinent to workers in the field of serological systematics since they must be certain that the sera or proteins or organisms used in their tests are unchanged from the native state, or if changes have occurred because of prolonged storage or other physical or chemical factors, they must be prepared to correct for it.

The sera of Crustacea contain one principal protein, hemocyanin (Allison and Cole, 1940). They are excellent antigens when injected into the rabbit. They are usually considered to be relatively pure systems. However, electrophoretic patterns (Cohn and Edsall, 1943), sedimentation constants (Dawson and Mallette, 1945; Redfield, 1934), and $(NH4)_2SO_4$ precipitation (Bolton, personal communication) all indicate that this single serum protein may be composed of several molecular "species" of hemocyanin. The hemocyanin molecules are large with molecular weight ranging from 300,000 upward to several millions as calculated by Svedberg and his collaborators from data obtained by using the ultracentrifuge methods of sedimentation velocity and sedimentation equilibrium. A glance at almost any titration curve of the hemocyanin antigen-antibody system (Fig. 3) will show irregularities and disturbances in the modality of the plot. Occasionally a titration will show two distinct modes. These variations from a single mode frequency curve have long been considered as evidence of the probable presence of more than a single kind of antigen or antibody in the system. It is quite likely that the above mentioned molecular "species" are responsible for the stimulation of more than a single principal kind of antibody in the rabbit serum. This, of course, does not preclude the possibility that a single molecular "species" could stimulate the production of two or more distinct kinds of antibodies. Until such time as this can be proven, it is simpler to assume that a single kind of antibody is produced against each kind of antigen, and that the appearance of additional modes in the titration curve of any antiserum is due to the reaction of individual molecular "species" in the antigen complex with their homologous antibodies. This assumption is not inconsistent with the observed behavior of antigen-antibody systems.

The serological identification of all proteins resides in their structural peculiarities and in their chemical nature, i.e., the kinds, proportions and arrangement of the amino acids and prosthetic groups all are believed to affect the serological activity of proteins. It is well known that mild treatment both chemical and physical, will alter the nature of proteins (Landsteiner, 1936; Cohn and Edsall, 1943). This fact presents a challenge to all investigators who are using animal proteins as representing the nature of the organisms with which he is working. Studies in serological systematics may require that animal sera be collected and saved, sometimes for a period of years, before numbers of different species sufficient for comparative in-

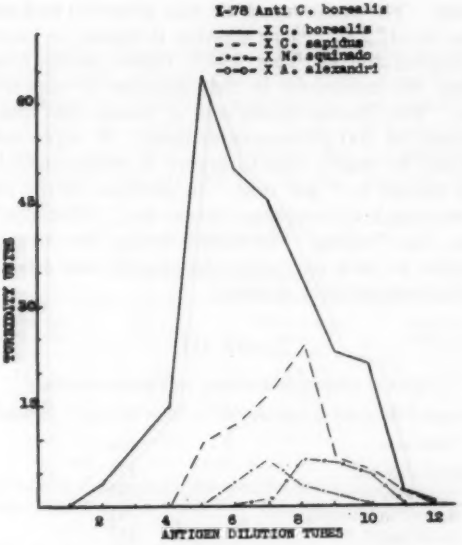

FIGURE 3. A typical series of precipitin titration curves showing an order of serological relationship among four crabs. The homologous reaction between *Cancer borealis* and the antiserum produced against it exceeds all others. Then the alignment occurs, in the order of their decreasing curve areas, *Callinectes sapidus, Acanthocarpus alexandri,* and *Maia squinado,* which is in accord with accepted systematic relationships.

vestigations are secured. Further, sera frequently have to be collected under field conditions, necessitating the use of preservatives. There are other aspects of the general problem of antigen comparability than those resulting from the chemical action of preservatives which must also be considered before comparisons of animal sera can be made with confidence. Following are the results obtained from attempts to test the affect of some of the variables encountered in the preparation of animal sera for comparative studies in serological systematics.

Physical treatment

Usual laboratory procedures have been to allow the blood to clot and the sera to be expressed for about 24 hours. The sera are then centrifuged at 2000–3000 r.p.m. for 20 minutes, filtered sterile in Seitz filters, bottled, and stored in the refrigerator at 3° ± 1° C. Any or all of these steps could alter the nature of the serum proteins and thus modify their serological activity. Table III summarizes a series of tests performed to examine the effects of treatment in the laboratory. The sample of blue crab serum designated in the table as *Callinectes sapidus* 47–2 represents the sera of 24 large male crabs all bled within one hour. The pooled collection of sera was divided into several parts and given various kinds of physical treatment in the laboratory. All of the antigens were tested against antiserum I–86 (Anti-*C. sapidus* 47–1) which was prepared against a fresh, sterile filtered

sample of crab serum. The antiserum I–86 was powerful and was diluted with 10 parts buffered saline at pH 7.0. Since dilution is known to increase the specificity of an antiserum (Boyden and DeFalco, 1943) under certain conditions it was advantageous to dilute the antiserum in this manner to magnify any differences among the antigens. The dilution factor was so chosen that total turbidities would summate in the vicinity of 300 galvanometer units. A curve area this large tends to minimize variations in results due to errors in experimental techniques. Experimental error is limited to 5 per cent. In addition to the treatments listed in the table attempts were made to lyophilize (freeze-dry) (Florsdorf and Mudd, 1935) parts of this sample, but "boiling" [3] occurred during the course of drying, which denatured the proteins to such an extent that insufficient amounts of them could be restored to conduct comparative studies.

TABLE III

Effect of laboratory handling on antigen reactivity

Antiserum I–86 Anti *C. sapidus* 47–1 (1 + 10) × *C. sapidus* 47–2

Treatment	Area	% Change
Centrifuged, filtered	279	0
Centrifuged, unfiltered	283	1
Uncentrifuged, unfiltered	287	3
Frozen, centrifuged	287	3
Frozen, uncentrifuged	290	4
Room temperature, centrifuged, filtered	275	1
Room temperature, centrifuged, merthiolate	285	2

The homologous antigen *Callinectes sapidus* 47–2 was centrifuged and sterile filtered before it was used as an antigen. Changes due to above listed treatments appear to be negligible.

It is realized that any tests involving comparisons with only a single antiserum prepared from but one kind of the possible antigen types present but a minimum of data on the comparability of antigens. Moreover, in the type chosen (Table III, explanation), it still is possible that the unfiltered, and the frozen samples could have constituents not possessed by the filtered samples. That there was a high degree of correspondence among all of the antigens tested is testimony to the fact that the unfiltered, and frozen samples contain corresponding antigens to those in the filtered material, also that filtration does not significantly reduce the quantity of such antigens nor the quality of their reacting (combining) capacity. For the short period of time (24 hours) involved between the beginning and end of the processing, the sample held at room temperature showed no difference from the others. A retesting of the room temperature sample seven days later also revealed no significant change in activity.

[3] This is the bubbling which occurs when the rate of sublimation of the frozen material undergoing desiccation is slow and thawing occurs at the inside glass surface of the containers because of the transfer of atmospheric heat to the frozen solid while the whole system is under vacuum. The relationships between the rate of heat intake from the atmosphere at the exterior glass surface of the containers, the rate of heat loss at the evaporating surfaces of the product and the rate of escape of water vapor from the product to the condenser is apparently upset.

Effect of cold storage

The question of antigen comparability is bound to the problems related to the storage of sera for prolonged periods. In order to examine adequately the effects of storage it was necessary to establish whether or not significant alterations occurred in samples of sera of various ages. Table IV summarizes the results of these tests. It is readily seen that no demonstrable immunological alteration occurred between the samples one day old (22 hours) and the other older samples.

TABLE IV

Antigen comparability

Sample	Number of individuals	Per cent protein	Whole curve area	Per cent deviation	Age of sample when tested
47–3	12	7.04	280.1	−0.3	22 hours
47–2	12	7.00	278.8	−0.1	1 month
*47–1	24	7.39	279.1	0.0	4 months
36–1	5	5.05	281.8	−0.9	11 years

Table showing the lack of change in serum samples of the blue crab, *Callinectes sapidus*, when tested with an antiserum against one of them (*). Samples stored at 3° ± 1° C.

The fact that the eleven year old sample number 36–1 of *C. sapidus* possessed a precipitinogen activity equal to the very fresh sera of this species is very surprising and important. This old serum has been kept at refrigerator temperatures practically continuously since it was collected in 1936. The amount of alteration that goes on in these serum proteins, if any occurs, must be very slight not to be detected by immunological testing. All the samples listed in Table IV represent pooled samples of the sera of five or more animals. It is conceivable, however, that individual variability of the sera does exist and that the pooling nullifies this variation, presenting for test a more or less "common denominator" kind of serum to the testing antiserum.

Serological variation

Interesting evidence of the stability and lack of variability among various samples of sera of the same species of spiny lobsters, *Panulirus argus*, is illustrated in Table V. Here samples collected over a period of years (1932–1939) were tested against an antiserum made to one of them. The test antigens were selected to reveal the differences due to aging, differences due to sex, and differences between single specimen samples and pooled samples containing the sera of more than one individual. The amount of difference in the serological reactivity among these different categories was negligible. In no instance did the variations in the whole curve areas exceed the experimental error of the method of testing. This is a remarkable fact, considering the length of time these sera have been stored; the youngest sample being nine years old at the time of testing, the oldest sample being fifteen years old.

Considering the sensitivity of the precipitin tests to slight alterations in chemical structure, the lack of variation in the amount and kind of reactivity demonstrated in the data of Tables IV and V is testimony first to the care in the laboratory prepa-

TABLE V

Antigen comparability

Sample	Number of individuals	Per cent protein	Whole curve area	Per cent deviation
32–1	"Pooled"	6.07	345.3	−0.2
32–2	"Pooled"	6.52	345.6	−0.1
34–C	1	4.55	351.6	1.5
34–D	1	5.33	340.8	−1.4
34–E	1	2.66	357.0	2.9
34–F	1	7.66	355.7	2.8
34–G	1	3.69	343.7	−0.6
36–1	1	5.31	345.2	−0.2
36–3 & 4	2	10.05	353.6	2.5
36–5 & 6	2	7.44	350.6	1.3
36–10	1	6.07	354.5	2.5
36–11	4	6.52	351.3	1.5
*39–1	5	4.59	345.9	0.0
39–3	9	6.02	349.4	0.2

Table showing the high degree of serological similarity among different samples of the spiny lobster, *Panulirus argus* collected between 1932 and 1939, and tested with an antiserum against one of them (*). Tests were conducted in 1947.

ration of the sera, and the use of a correct method for maintaining useful samples for long periods of time, and second, a testimony to the durability and stability of hemocyanin proteins when collected and preserved in the manner described above.

Color changes

Hemocyanin proteins possess a copper radical and in the oxidized state and in vitro have a blue to green color when in solution. Occasionally, variations have been noted in the color of the sera after they have been sterile filtered and bottled. The most frequent variation is a change from the blue-green color to dark brown. This change is due in part to the free carbon on the Seitz pads which forms there as a result of over-heating during sterilization of the filter, and also to chemical changes in the proteins themselves since the color change has been noted not only during the filtration processing but also in some samples previous to filtering. The brownish color does not appear to play any role in altering the reactivity of the sera since these samples compare favorably with others of the same species possessing the blue-green color.

If a sample of hemocyanin serum is contaminated with bacteria, these organisms flourish and the blue-green, oxidized condition of the serum changes to a colorless liquid, with a cloudy suspension of bacteria. If such sera are kept in the refrigerator, even though contaminated, no recognizable alteration occurs in the activity of the dissolved protein when tested against an antiserum. Removal of the bacteria by filtration or centrifugation or both quickly restores the oxidized condition of the serum. Its serological behavior seems not to be altered even when the serum has been stored in the contaminated state in the refrigerator for prolonged periods.

Precipitates in vials

Another phenomenon that has been seen to occur in sterile, bottled sera is the appearance of precipitates in the vials. In some instances the amount of material coming out of solution has been considerable. The supernatants of many of these vials were examined and found still to contain sufficient protein in solution to warrant testing for comparability with nonprecipitated samples of the same species. Surprisingly enough the still soluble fractions of the total proteins in the antigens apparently possessed all of the reactivity characteristic for the serum. This would indicate that the precipitate is denatured protein which has not undergone any appreciable decomposition. The presence of large amounts of free amino acids and peptides in solution above the precipitates would present free radicals that might combine with the antiserum, blocking the reaction and causing some difference in the amount of reaction observed to occur between the still soluble protein and the antiserum. This is not the case with these systems. The precipitates, moreover, still possess the capacity to combine with antisera, as saline suspensions of them readily reveal. The antigen precipitates go back into solution readily using dilute alkali, but not in saline solutions up to 1.7 per cent NaCl.

Serological Systematics of Some Crustacea

Using the procedures described above, it thus appears feasible to collect and store the sera of Crustacea for long periods of time (15 years in the data given) without significant alteration in their specific properties. Comparative studies of the various species of Crustacea become in effect analysis of the biochemical nature of the organisms concerned based on the quantitative comparison of the nature of their serum proteins, as reflected by turbidities developed in the antigen-antibody reaction.

Erhardt (1929) reviewed the early work done on serological systematics among the Crustacea. Some of these early investigators obtained results which do not agree with systematic classifications based on morphological and embryological data. In one instance Nuttall and Graham-Smith, each using the same antiserum, compared exactly the same organisms and obtained very different results. In most other instances the serological work generally agreed with well established classifications. Most all of these early tests were done using the interfacial or "ring" test method for comparisons. The correspondence of techniques among the various workers ended there because the antigens were not standardized and because results of tests based on antiserum titers and those based on antigen titers cannot be directly compared.

Boyden (1942) pointed out the deficiencies and inadequacies in these early serological investigations and in two papers (1939, 1943) presented the first really quantitative study in the serological systematics of the Crustacea. He compared representatives of five families of the Brachyura and two species of the Macrura. It was in the later paper that the concept of the "serological yardstick" was introduced. The relationships of the sera of species of the same genus, and genera of the same family, and different families of the tribe Brachyura were remarkably consistent in their serological values. All of his tests were made using the photronreflectometer and standard procedures as outlined by Boyden and De-

Falco (1943). The inconsistencies apparent in earlier investigations by other workers were not observed to occur in these studies.

The availability of a larger and more representative group of Crustacea made an extension of Boyden's work possible. Table VI summarizes the serological comparisons made. It should be pointed out that the values given are averaged from two or more tests. The generally higher interspecific, intergeneric and interfamily relationships than those reported by Boyden seem to be due to the use of the presensitization technique to produce more powerful and less discriminating antisera.

From Table VI it is apparent that the antisera differ in their capacities to discriminate among the families of Brachyura. It appears that the Portunidae, Xanthidae and Cancridae are more closely related to each other than they are to the Ocypodidae, Calappidae and the Majidae. Of special interest is the species *Geryon quinquedens* here listed as a member of the family Goneplacidae, in accordance with Rathbun's classification (1937). She concluded that Goneplacidae were closely related to the Xanthidae. Bouvier (1940) places Geryon in the family Xanthidae. The serological tests indicate a degree of correspondence for *Geryon* which is approximately the same in heterologous reactions as for the xanthid species. Further critical testing is necessary to definitely establish the affinities of *Geryon*.

TABLE VI

The relative relationship among Crustacean species representing seven families in the tribe Brachyura

Antisera	Antigens (Relationship in per cent)												
	Callinectes sapidus (Portunidae)	*Callinectes marginatus* (Portunidae)	*Carcinus maenas* (Portunidae)	*Cancer borealis* (Cancridae)	*Cancer irroratus* (Cancridae)	*Cancer pagurus* (Cancridae)	*Cancer anthonyi* (Cancridae)	*Panopeus herbstii* (Xanthidae)	*Menippe mercenaria* (Xanthidae)	*Ocypode albicans* (Ocypodidae)	*Acanthocarpus alexandri* (Calappidae)	*Geryon quinquedens* (Goneplacidae)	*Maia squinado* (Majidae)
Callinectes sapidus (Portunidae)	100	78	46	33	44	37	33	24	48	18	12	38	8
Cancer borealis (Cancridae)	29	22		100	75	54		20	42	9	8	22	9
Cancer irroratus (Cancridae)	49	46	33	78	100	73	72	30	35	43	10	31	30
Menippe mercenaria (Xanthidae)	27		21	24	21	16	24	37	100	8		27	3
Ocypode albicans (Ocypodidae)	30		40	33		34		39	34	100	39	6	33
Acanthocarpus alexandri (Calappidae)	12		13	15		15				18	100	14	2
Geryon quinquedens (Goneplacidae)	15			13					15		8	100	9
Maia squinado (Majidae)	21			16					13	13	8		100

From Table VI it is readily seen also that species within a given genus react with each other to a greater extent than with any other organisms.

The problem of establishing in detail interfamily relationships among Crustacea by serological methods is one that would entail the production of large numbers of antisera in an attempt to secure serological reagents which are sufficiently powerful to react significantly with the more distant families and at the same time discriminate among the representatives of these families sufficiently to establish a verifiable order of relationship.

Summary

Serological Systematics is a branch of Serology concerned primarily with the classification of organisms. The taxonomic characters usually concerned are the serum proteins of organisms. The natural relationships obtained are those revealed by an antiserum in combination with its homologous antigen and various heterologous antigens which react in proportion to their degrees of correspondence to the homologous antigen. Many factors influence the antigen-antibody reaction. A few conditions which are of importance to studies in systematic serology have been investigated and the results given.

Antigens tested for comparability under a variety of the circumstances met in ordinary laboratory handling such as freezing, filtration and centrifugation, showed no significant deviation from each other in their serological activity. Age, within the limits stated, was shown to have no effect on the serological activity of serum antigens which are sterile filtered and stored just above freezing. Antigens in cold storage for as long as 15 years had the same activity as freshly prepared samples. Pooled serum antigens showed no serological differences from the sera of individuals. No differences were demonstrated in sera due the sex of the organisms. For cold stored antigens color changes in the vials did not indicate alteration of the reactivity of the proteins. The remaining soluble portions of protein in vials showing apparently spontaneous precipitation of the protein gave the same reactivity as freshly prepared antigens. Bacterial contamination, if not permitted to endure too long, and if kept under refrigeration, does not alter the proteins significantly. The reconstitution of lyophilized hemocyanin sera for serological testing was not successful.

The studies in systematic serology have been extended to include new families of decapod Crustacea. For the species of Brachyura tested it appears that the families Portunidae, Xanthidae, and Cancridae are more closely related to each other than all of them are to the Ocypodidae, Calappidae and Majidae.

Further studies into the conditions which may modify the serological reactivity of proteins are needed. Continuing investigations, examining both the validity of the methods used in measuring antigen-antibody reactions and the methods used in the preparation of these primary serological reagents, are necessary components of truly critical studies in serological systematics.

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THE GROWTH AND METAMORPHOSIS OF THE ARBACIA PUNCTULATA PLUTEUS, AND LATE DEVELOPMENT OF THE WHITE HALVES OF CENTRIFUGED EGGS

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The pluteus of *Arbacia punctulata* which is well known to many investigators is the early pluteus with four arms, a pair of long anal (post-oral) arms on the ventral side, and a pair of short oral (pre-oral) arms on the dorsal side. In order to obtain further development of the pluteus, it is necessary, at Woods Hole, to feed the animals a rather special diet. In 1882 W. K. Brooks and two of his students, Garman and Colton, raised the plutei of *Arbacia punctulata* at Beaufort, N. Carolina, apparently without any special feeding. The sea water there is rich in diatoms, and the plutei can probably obtain what they require for growth from the sea water. This work was published by Brooks (1882) in his Handbook of Invertebrate Zoology, and by Garman and Colton (1883). The drawings are excellent for detail, but there is no indication of size and not adequate indication of age. A few stages had previously been described and figured by Fewkes (1881) working in A. Agassiz's laboratory at Newport, R. I. The present account with photographs gives the development of the pluteus with regard to size, sequence of events and rate of development at Woods Hole.

The best food for sea urchin larvae has been found to be the diatom, *Nitzschia closterium;* I found that they would also grow on the diatom, Licmophora. The Nitzschias themselves require a special diet, and must be raised in pure culture; they are raised on Miquel's solution.[1] The method has been worked out by Allen and Nelson (1910) in Plymouth, England, and has been used by many investigators at the Plymouth laboratory. Shearer, deMorgan and Fuchs (1914) have in this way succeeded not only in raising the normal plutei of several species of sea urchin to maturity, but have also raised some hybrid plutei to maturity. Fuchs (1914) has even obtained the next or F_2 generation of these hybrids. Unfortunately, the late

[1] Miquel's solution, as modified by Allen and Nelson (1910), consists of:

Solution A	KNO_3	20.2 grams
	Distilled water	100 cc.
Solution B	$Na_2HPO_4 \cdot 12\ H_2O$	4 grams
	$CaCl_2 \cdot 6\ H_2O$	4 grams
	$FeCl_3$ (melted)	2 cc.
	HCl (concentrated)	2 cc.
	Distilled water	80 cc.

To each liter of sea water add 2 cc. Solution A and 1 cc. Solution B, and sterilize by heating to 70° C. When cool, decant off the clear liquid from the precipitate, which will have formed when Solution B is added to the sea water.

Ketchum and Redfield (1938) have used a slight modification.

larval characters of *Echinus esculentus* × *E. acutus* from which the F_2 generation was obtained are alike in the two species, so that no information as to inheritance could be obtained, and none of the F_2 hybrids between *E. esculentus* or *E. acutus* × *E. miliaris* which would have given the information, reached maturity. Miss Gordon from MacBride's laboratory raised some Arbacia plutei at Woods Hole in 1926, using this method, but she was particularly interested in the later development of the test, and gives no account of the changes in the pluteus in her publication (1929).

There are two forms of *Nitzschia closterium* both of which are devoured by the plutei. One, the large form (Plate I, Photograph 1) is about 100 μ long; the other, forma *minutissima* (Photograph 2), is about 24 μ long and is the variety used in the Plymouth laboratory. The Nitzschias are swept into the oesophagus and stomach (Photographs 3, 4) by cilia.

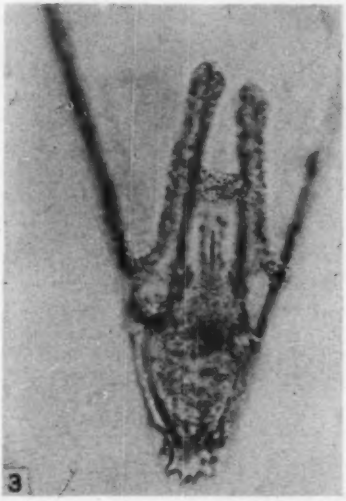
Several stages in the early development from the fertilized egg are shown on Plate II (Photographs 1–6). The micromere stage (Photograph 2) is the first sign of differentiation of cells, the micromeres being small and colorless. It also marks the beginning of asynchronous cleavage; in Arbacia there is a definite 12-cell stage preceding the 16-cell stage. With further cleavages a blastula is formed and emerges from the fertilization membrane (Photograph 3) in about 8 hours, the time varying by one or two hours in different batches and with different temperatures. At this stage I have estimated that there are 1,000 to 2,000 cells representing 10 to 11 cleavages (2^{10} to 2^{11}). The blastocoel becomes larger, leaving a single layer of peripheral, ciliated cells (Photograph 4). Then invagination takes place (Photograph 5), and a gastrula is formed (Photograph 6). At this time the skeleton appears in the form of triradiate spicules, one on each side of the gut. During this period there is no appreciable increase in size of the organism over that of the egg (without the fertilization membrane), and one would not expect an increase before the alimentary canal is complete and it can take in food from the outside. Now growth occurs and differentiation into the pluteus form with skeletal rods on each side (Photograph 7). At this stage the large pigment spots characteristic of the later plutei begin to form. The young pluteus increases in size and the arms begin to grow out (Photograph 8). The pluteus is well formed in 24 hours (Photograph 9). It is larger on the second day (Photograph 10), usually reaching a maximum in three or four days (Photograph 11). The long anal arms may measure 410 μ from base to tip. Without special feeding, the pluteus may live three or four weeks, gradually getting smaller by resorption of its arms (Photographs 12–14). It has apparently obtained sufficient food for growth from the sea water for the first four days, but then requires additional food. The structure of a two day pluteus is shown in serial photographs (Plate I, Photographs 5, 6, 7). These are taken at different levels through the animal, corresponding to serial sections of imbedded material.

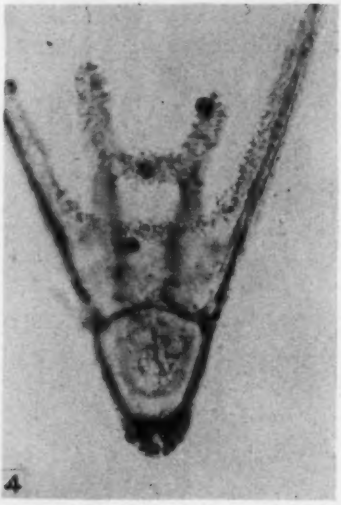
Further development of the three or four day pluteus may take place if *Nitzschia closterium* is added to the cultures of plutei. Only a few plutei in any culture continue to develop. The British investigators have found it expedient to have only a few individuals in a large amount of water, 20 to 30 in a half-gallon jar. (See MacBride 1914, p. 506.) I raised them in Syracuse watch glasses holding about 15 cc. of sea water with about a dozen plutei in each dish. The developing larvae

PLATE I

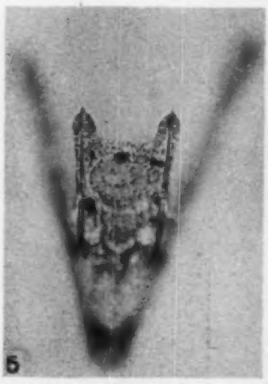
Nitzschia closterium

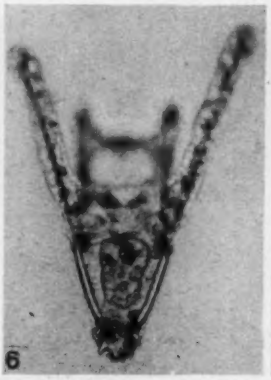
Nitzschia closterium forma minutissima

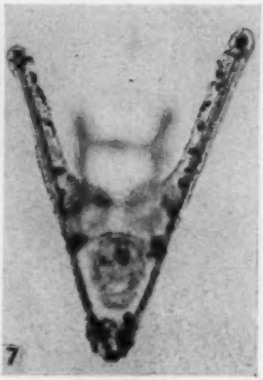




Plutei engulfing Nitzschias





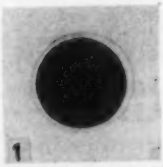


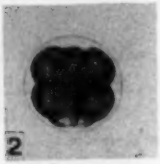
Serial photographs of 2 day pluteus

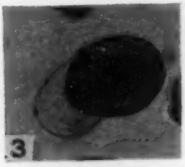
Dorsal Median Ventral

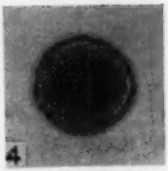
PLATE II

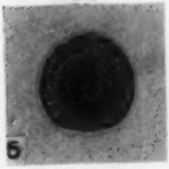
DEVELOPMENT WHEN NOT FED

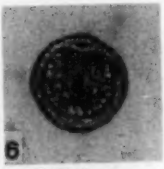

1
Fertilized egg


2
2½ hrs.


3
8 hrs.


4
12 hrs.


5
15 hrs.

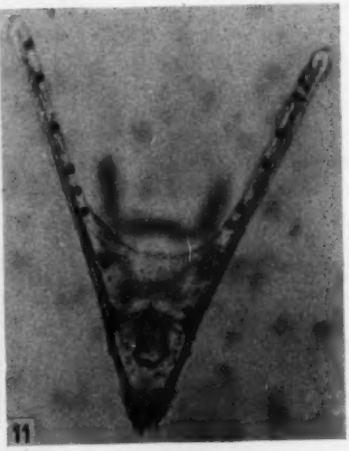

6
17 hrs.

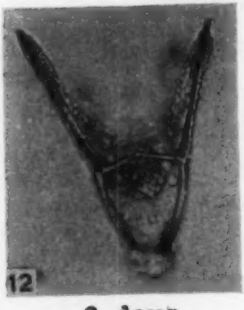
7
19 hrs.

8
21 hrs.

9
1 day

10
2 days


11
4 days


12
6 days

13
9 days

14
13 days

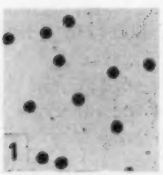
were transferred to fresh sea water with a pipetteful of the Nitzschia culture every few days. The eggs from which the final small adults were obtained were fertilized on July 12th, 1948, at Woods Hole, and taken to Princeton, N. J., on October 3rd. The last four died after completing metamorphosis on November 17th, a little over four months old.

The photographs on Plates III and IV, 1–18 are all to the same scale. The development varies in time in different batches, and in individuals of the same batch, so that the times given are only approximate. The eggs at this magnification (approximately 24 times) are of the size shown in Photograph 1. The one and the three day plutei to this scale are shown in Photographs 2, 3, shown with a larger magnification in Photographs 9, 10, 11 on Plate II (approximately 180 times). For the first four days, the development is the same whether fed Nitzschia or not, the food in the sea water being adequate. When fed, in about a week (Photograph 4, Plate III), the anal arms have become considerably longer. Then little knobs appear toward the base of the animal, which, by the eleventh day have grown out into a definite pair of new arms, the ventral lateral or postero-lateral (Photograph 5). These are always heavily pigmented at the tips which appear very red. These grow longer and a pair of knobs appear between the original anal arms and the new red-tipped arms. These are slightly noticeable in Photograph 6. In a month's time, these have grown into a second new pair of arms (Photograph 7); these are the dorsal lateral or postero-dorsal arms. These arms usually do not have red tips, although sometimes all the arms are pigmented at the tips. All the arms become continually longer (Photograph 8). The animals are now easily visible to the naked eye and look like small spiders. The arms are variable in length individually and relatively to each other. They are all ciliated, the cilia on the red-tipped ones being much longer and stronger than those on the other arms. The animal now swims actively by means of its cilia, and also walks or tumbles about on the tips of its arms, which can be readily moved. The arms are quite fragile and are easily broken off when the animal bumps into something or when it is transferred to another dish. They have great regenerative capacity, the arms growing out again when broken off. One pluteus, from which I had cut off the red-tipped arm about half way down, had completely regenerated it together with the red pigment in five days so that it then looked exactly like its mate. The broken piece may seal itself off and swim about actively by means of its cilia like a complete organism, looking something like a paramoecium (Plate VI, Photograph 6). One of these pieces was alive and active for four days when it was inadvertently lost. The body of the adult Arbacia is seen as a yellowish green mass in the pluteus, the dark area in Photographs 7, 8 (Plate III) and thereafter. There are areas of dark red pigment on the surface. The young adult is formed in the body of the pluteus and grows at the expense of the pluteus.

By six weeks the pluteus has become quite complicated, with two pairs of secondary oral arms, and four tubular processes (auricular lobes), two dorsal and two ventral. In Photograph 9, one sees several of the oral arms (at the anterior end) and a pair of the tubular processes (at the posterior end). A diagram of this stage is given by Miss Gordon (1929, p. 291). Soon after this, the five primitive ambulacral feet appear at one side of the body; these have suckers at their extremities and are continually expanded and contracted (Photograph 10).

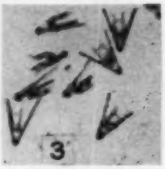
PLATE III

DEVELOPMENT WHEN FED



Eggs to scale

1 day

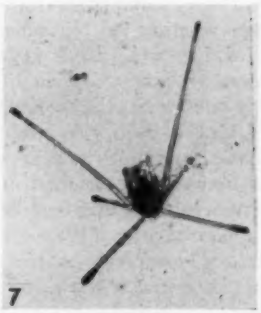


3 days

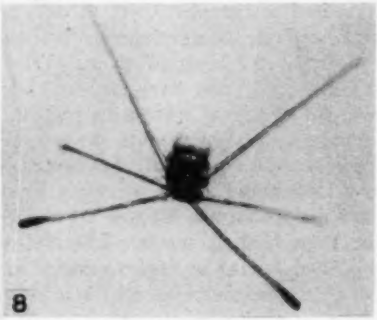
1 week

11 days

3 weeks

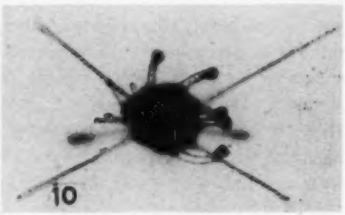


1 month

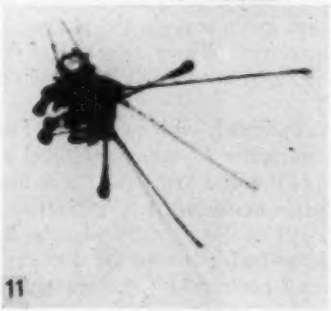


5 weeks

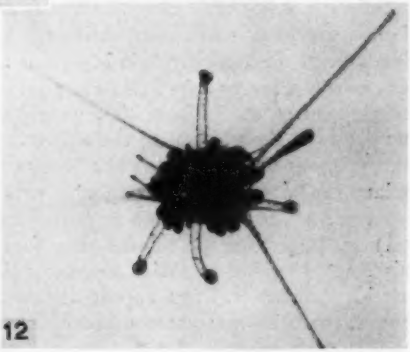
6 weeks



2 months



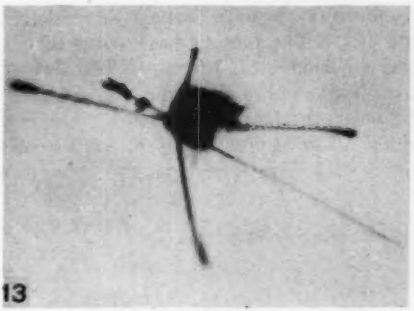
2 months



2½ months

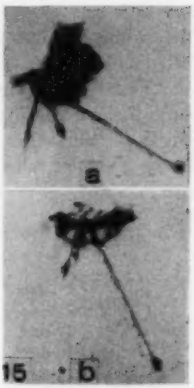
PLATE IV

DEVELOPMENT WHEN FED II



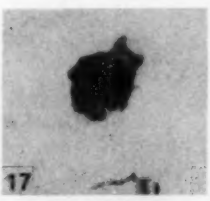
3 months

4 months

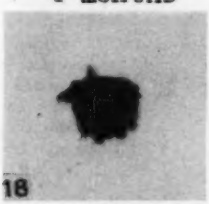


4 months

4 months



4 months

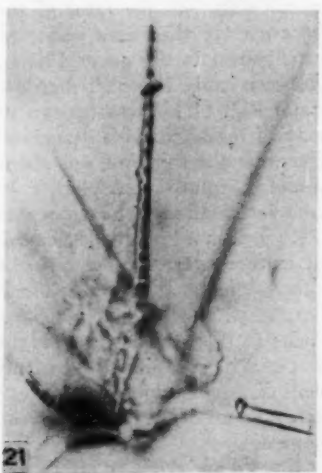


4 months

ARM SKELETON

Original

First new



Second new (middle)

In Photograph 11, also a two months pluteus, one sees the three pairs of long arms, two pairs of the short arms near the head of the pluteus which is still prominent, the five ambulacral feet, with suckers, and the body of the developing adult. After two or more weeks, between each two ambulacral feet are formed three flattened plates, the first set of spines (Photograph 12). The pluteus has now reached its full development and the arms their maximal length, ca. 1.6 mm. The length of the arms is quite variable in different individuals even of the same batch. To give some idea of the increase in length of the arms, the following table is given of the length of the long anal arm at different ages. The figures represent measurements of the average better developed ones, the poorly developed ones not being taken into account.

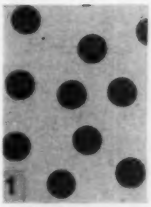
Approximate length of long (anal) arm from base to tip (μ)

	Fed	Not fed
1 day	180	180
2 day	300	300
3 day	380	380
4 day	410	410
5 day	450	330
6 day	480	250
1 week	600	200
11 days	700	180
2 weeks	750	150
3 weeks	800	
1 month	1000	
1½ months	1300	
2 months	1400	
2½ months	1600	

After reaching their maximal size, and often earlier, the arms begin to go to pieces; the flesh peels off, leaving the bare skeleton (Plate IV, Photograph 13). By four months several of the arms have gone to pieces; the body of the adult is conspicuous, but the head of the pluteus remains (Photograph 14). Sometimes the arms are shed as a whole piece like a shell. In Photograph 15 three of the arms shown in the upper Photograph (a) were shed as a unit the following day, as shown in the lower Photograph (b). The animal was left with one arm (Photograph 16). This was thrown off, but the head of the pluteus remained, and the body of the adult with its primitive spines was developing (Photographs 17, 18). The animal at this time was about a half millimeter in diameter. These later stages in metamorphosis took place in my cultures within a few days. The whole process from fertilization to metamorphosis took over four months (July 12 to November 17). I do not know whether this is the normal period under natural conditions as my cultures were subjected to changes in temperature and food. My last four animals all died at this time. They are known to require a different food after metamorphosis, which I did not have at hand. According to Shearer, deMorgan and Fuchs (1914), the best food is the calcareous protozoan Trichosphaerium; and later the red alga Corallina, these furnishing the calcareous matter needed for the development of the test and spines (l.c., p. 276). Miss Gordon (1929) has given a complete account of the further development of the young adult with especial attention to the test.

PLATE V

PLUTEI FROM NORMAL EGG


1

Eggs to scale

2

3 days

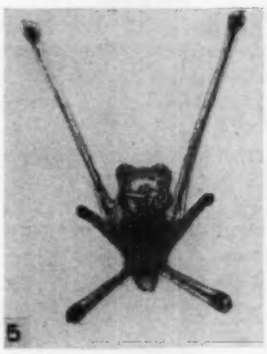
3

10 days

4

11 days

NORMAL


5

3 weeks

PLUTEI FROM WHITE HALF EGG

6

3 days

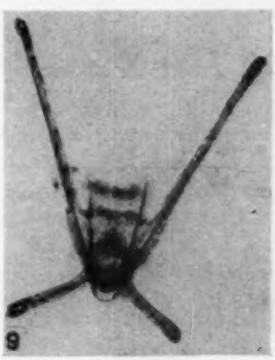
7

10 days

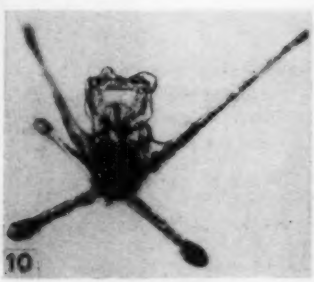
8

10 days

PLUTEI FROM WHITE HALF EGG

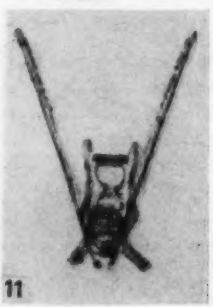

9

11 days


10

3 weeks

PLUTEUS FROM CENTRIFUGED EGG

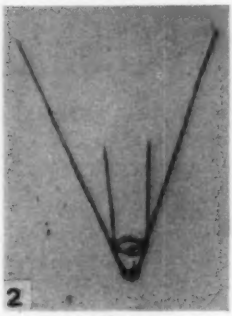

11

2 weeks

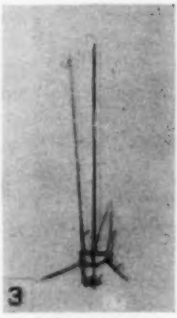
PLATE VI

SKELETONS

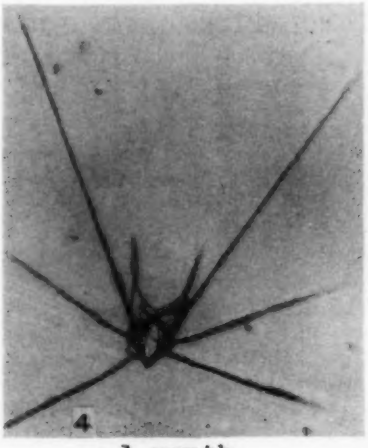
2 days



8 days



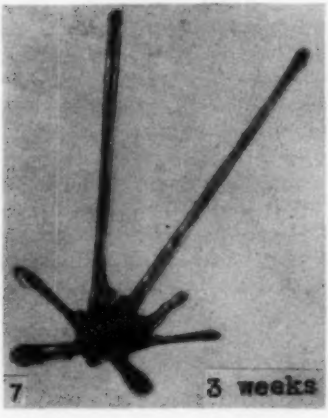
11 days



1 month

White half

ABNORMAL



BROKEN OFF ARM

The arm skeletons of the pluteus are interesting. The skeleton of the original long pair of (anal) arms is fenestrate, that is, it is a rod with holes in it (Plate IV, Photograph 19). The new pair of red-tipped (ventral lateral) arms have a solid skeletal rod with no holes (Photograph 20). This photograph shows also the concentration of pigment at the tip. The rod in the second new pair of long (dorsal lateral) arms, between the other two long pairs, is again fenestrate (Photograph 21). As is well known, the skeletons of the original long (anal) arms are solid in the plutei of many sea urchins. We have, in general, two types of plutei, those with fenestrate arm skeleton such as Arbacia, Tripneustes, Sphaerechinus and the sand-dollar, Echinarachnius; and those with solid arm skeleton, such as Strongylocentrotus, Lytechinus and Psammechinus. This has been of great value in hybridizing experiments, in determining maternal and paternal inheritance.

Development of the white half egg

The Arbacia egg can be separated into a white and a red half by centrifugal force. The white half when fertilized develops in the same way as the normal whole egg (E. B. Harvey, 1932, 1940). A normal pluteus is formed in two days similar to that from the whole egg except that it is smaller and lacks pigment. The pigment spots, however, begin to come in on the third day after fertilization, and continually increase. The white plutei, like those from the whole egg, do not develop beyond the four armed stage without special feeding. The photographs on Plate V were taken at a greater magnification than the preceding series on Plates III and IV (i.e. approximately 60 times). The eggs to scale are shown in Photograph 1; the normal three day pluteus in Photograph 2. Several later stages of normal plutei are shown in Photographs 3, 4, 5, for comparison with the same stages of the white plutei (Photographs 6–10). The ten day white plutei (Photographs 7, 8) are in all respects like those from whole eggs (Photographs 3, 4). There is the same massing of red pigment in the tips of the first new pair of ventral lateral arms. There is now no difference in size between the plutei from the half egg and those from the whole egg, in fact the former may be larger (Photograph 9). The three weeks pluteus from the half egg (Photograph 10) has the three pairs of long arms like that from the whole egg (Photograph 5). These were not carried any further, but it seems certain that the later development would be like that of the pluteus from the whole egg.

Development of the centrifuged egg

The very young pluteus from the centrifuged egg has the pigment granules concentrated in certain areas, most frequently above the mouth, though they may be in other positions. The pigment spots are also unevenly distributed at first, but after three or four days they are fairly uniformly distributed so that one cannot distinguish between the plutei from centrifuged eggs and those from normal eggs.[2] The later

[2] The pigment spots of the plutei are bright red, in contrast to the brownish red color of the unfertilized eggs. However, in centrifuged eggs, the concentrated mass of pigment granules is bright red. This difference in color is well shown in kodochrome slides when the contrasting objects are taken on the same slide. E. G. Ball (*Biol. Bull.*, **97**: 231) has compared the absorption spectra of acid alcohol extracts of plutei and eggs, and has found the two pigments identical.

development, when fed, is like that of the normal pluteus. In Photograph 11 is shown a two weeks pluteus from a centrifuged egg, which is similar to the pluteus from a normal egg of about the same age shown in Photograph 4.

Skeletons

The skeletons from plutei of various ages are shown in the photographs on Plate VI; the fully formed skeleton is shown in Photograph 4. A skeleton from a white half egg is shown in Photograph 5, quite like that from a whole egg of the same age (Photograph 3).

Abnormalities

The only abnormal later pluteus occurring in my cultures is shown in Photograph 7, on Plate VI. It had one extra arm on one side.

Magnification of Photographs

The photographs were taken of the living animal with different objectives, as indicated below in brackets, and a 10X ocular.

Plate I. Photographs 1, 2 about 420X (70X). Photographs 3, 4 about 240X (40X). Photographs 5, 6, 7 about 180X (30X).
Plate II. All photographs about 180X (30X).
Plate III. All about 24X (4X).
Plate IV. Photographs 13–18 about 24X (4X). Photographs 19–21 about 240X (40X).
Plate V. All about 60X (10X).
Plate VI. Photographs 1–5 and 7 about 60X (10X). Photograph 6 about 240X (40X).

Explanations of the plates are given on the plates, and in the text.

Summary

1. The growth and metamorphosis of the *Arbacia punctulata* pluteus after the four day stage has been traced by means of photographs. The plutei were raised on the diatom, *Nitzschia closterium*.

2. The pluteus from the white half egg develops pigment spots after the third day, and develops in exactly the same way as that from the whole egg, when fed Nitzschia.

3. The pluteus from the centrifuged, stratified, egg becomes like that from the normal egg after the third day and develops similarly when fed.

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A NOTE ON THE REORIENTATION WITHIN THE SPINDLE OF THE SEX TRIVALENT IN A MANTID

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In most mantids during the formation of the spindle in late meiotic prophase, the kinetochores of each bivalent move suddenly apart towards opposite poles stretching the chromosomes. This is known as the premetaphase stretch stage and is followed first by recontraction of the chromosomes and only then by their gradual congression at the equator to form the metaphase plate. The bivalents move to the equator with no change in their original orientation of one kinetochore to each pole. The behaviour of the sex trivalents, however, both during the stretch stage and the ensuing congression, presents a more complicated situation. As normal behaviour one would expect the X_1X_2 kinetochores to orient towards one pole and the Y kinetochore towards the other, but actually a large number of sex trivalents appear to orient at random. There result, in addition to normal orientation, several types of malorientation. White (1941) first observed such malorientation of sex trivalents; he noted a high frequency of malorientation in 3 species of mantids and concluded that some reorientation must take place before metaphase formation. The conclusive proof for such reorientation was given by Hughes-Schrader (1943), who demonstrated, in *Stagmomantis carolina*, a decrease in the number of maloriented sex trivalents between premetaphase stretch and final metaphase. In the present note are recorded observations on *Hierodula* sp. which show that in this species also reorientation of trivalents takes place.

The material consists of testes fixed in PFA3, from a nymph of *Hierodula* sp., collected near Bombay (India), and placed at my disposal by Professor J. J. Asana to whom I am greatly indebted. Sections ranging from 6 to 10 μ and stained in iron haematoxylin were used for the study.

The presence of a sex trivalent in this species of Hierodula (specific identification is not available) was recorded by Asana (1934) who also established the total number of chromosomes in the male as 27. Later Oguma (1946) recorded the same chromosome complement in the males of four species of Hierodula.

In the present material all sex trivalents in which a lateral view of the spindle is presented during the premetaphase stretch, and again during the metaphase were counted. Those trivalents whose position prevented a positive determination of their orientation were also recorded. During the stretch stage various types of orientation were found. In some cases one of the X's was oriented towards one pole while the other X and the Y were oriented towards the opposite pole; in others the Y was stretched between the two X's while in the rest the orientation was normal. These configurations, assumed by the sex trivalents during the premeta-

* This work was done in the Department of Zoology, Columbia University. I am greatly indebted to Professor Franz Schrader for giving me the facilities to work and to Dr. Sally Hughes-Schrader for suggesting this problem and her helpful criticism.

phase stretch, involve a genuine orientation of the kinetochores to the division center comparable to that ordinarily occurring at metaphase. This is shown not only by the position of the chromosomes and their attenuation at the kinetochores but also by the fact that chromosomal fibers are formed between the center and the kinetochore in both maloriented and normally oriented chromosomes.

TABLE I

Orientation of sex trivalent during premetaphase stretch and at metaphase

	Normal	Malorientation			Not analyzable
	X_1X_2 ↕ Y	X_1 ↕ Y ↕ X_2	X_1Y ↕ X_2 X_2Y ↕ X_1	Total	
Premetaphase	42 = 65.6 per cent	15	7	22 = 34.4 per cent	36
Metaphase	149 = 97.4 per cent	3	1	4 = 2.6 per cent	0

It will be seen from Table I that during premetaphase stretch as many as 34.4 per cent of all analyzable sex trivalents are maloriented. This number is, however, strikingly reduced to 2.6 per cent in metaphase. Even if all non-analyzable trivalents were assumed to be normally oriented, still the number of maloriented sex trivalents is 22.0 per cent of the total, which is quite significant in relation to the 2.6 per cent of malorientation found at metaphase. This clearly proves the occurrence of reorientation in Hierodula and supports the earlier observations of White and Hughes-Schrader. This conclusion leads us to the basic question of what processes underlie the reorientation. The probable explanation must be sought in the role of the kinetochore. Further investigation of this problem in other species of mantids is planned.

SUMMARY

During the stretch stage in the meiosis of the male Hierodula a high percentage of malorientation of the sex trivalent is found. At metaphase, however, the number of maloriented configurations is so small that a considerable amount of reorientation must occur between these two phases. The forces involved are obviously of some significance in the general problem of the mitotic mechanism.

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MODIFICATION OF THE RESPONSES OF TWO SPECIES OF BUGULA LARVAE FROM WOODS HOLE TO LIGHT AND GRAVITY: ECOLOGICAL ASPECTS OF THE BEHAVIOR OF BUGULA LARVAE

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It is well known that the distribution of sessile organisms is markedly influenced by environmental conditions that affect the setting of their larvae. Thus, oysters are abundant at the mouths of certain rivers where their copper-laden waters are mingled with that of the ocean, and both maxima and minima of settings can be correlated with the amount of copper present during a critical stage of their development. (Cf. Prytherch, 1934.) Furthermore, rather frequent cases of co-occupation of a habitat by communities of sessile organisms of entirely different phyla would suggest the possibility that conditions favorable for the setting of one group are also advantageous to the other. The following observations on the effects of light and temperature on two species of *Bugula*, *B. flabellata* and *B. turrita*, from the Woods Hole region are presented partly because these factors influence distribution by affecting the attachment, metamorphosis and growth of the larvae and partly for the purpose of comparing and contrasting the behavior of these organisms with that of *B. neritina*, formerly studied at Beaufort, North Carolina (Lynch, 1947). The problem of distribution of two species of the same genus is often a baffling one, as is well illustrated by the fact that *B. flabellata* and *B. turrita* occupy communicating waters not more than 100 yards from each other yet each is found almost exclusively in its own particular habitat. A better understanding of the physiology of the larvae may lead to the beginning of a solution of such problems of distribution.

Contrasting features of the larvae of B. flabellata and B. turrita

Since a description of the larva of *B. flabellata* and its reactions to light and gravity has been given by Grave (1930), only contrasting features of the two larvae or additional details of their behavior will be presented here. *B. flabellata*, the smaller of the two (average, 0.17 by 0.19 mm.), has ten or twelve flagella in its pyriform region and is devoid of light-reactive organs. *B. turrita* is larger (average, 0.19 by 0.20 mm.), has four or five long slender flagella and four brilliant-red, spherical eye-spots, two very close to the pyriform organ and two slightly larger ones located in the opposite hemisphere.[1] The whole body of the larva, except the eye-spots fluoresces faintly in ultra-violet light of 3600 Å. Ejected holdfast material and disintegrated larvae, however, do not fluoresce. This response to

[1] It is difficult to understand why Grave (1930) referred to the light-receptive organs of the larva of *B. turrita* as being "darkly pigmented" and failed to mention their red color. Even with the light cut down to a minimum, their brilliance is an outstanding feature.

ultra-violet light seems to be caused by some substance in the integument of the larva. The cilia at the equator of both species are more active than those in other regions.

The taxonomy of the genus *Bugula* leaves much to be desired at the present time. In the position, color and shape of the eye-spots and in external structure the larva of American *B. turrita* is identical with that of the European *B. plumosa,* but the ground color of the former is yellow or flesh-colored (like that of the European *B. flabellata*), with a faint band of orange pigment at the equator, whereas that of *B. plumosa,* according to Nitsche (1870), is pure white. Furthermore, the larva of European *B. flabellata* differs from the American form, devoid of light receptive organs, since the former has ten symmetrically arranged eye-spots that are figured by Nitsche (1870) as slit-like or elongated and surrounded by fine cilia, but are ovoid according to Barrois' (1877) plates. Grave (1930) noted the difference between American and European species and stated that Calvét (1900) also referred to a confusion of varieties. Is the organism called *B. turrita* in America merely a variety of *B. plumosa?* Or is this a case in which evolutionary changes have affected only the larvae in some instances and only the adults in others? Since two species of *Bugula* sometimes have identical larvae and since the same species apparently may have two different larval forms, it is not unreasonable to suppose that mutations could affect the form of either larva or adult independently of each other.

Materials and Methods

Adult colonies were kept in darkness over night and until the experiments were begun. After exposure of the parental colonies to light, the photopositive larvae released by illumination were easily pipetted to experimental vessels. Generally a single group of adult colonies yielded enough larvae for experiments on several different days. For observations on the geotropism of the larvae and their reactions to light, small vials 1.5 cm. in diameter and 8 cm. high, as well as stender dishes were employed; for microscopic examinations stender dishes and occasionally well slides were used. When slides were used, they were covered to prevent evaporation, which hastens setting by increasing the salinity. A box, 5 by 5 by 15 cm., covered with black paper on all surfaces except the exposed side, was used for experiments on the effects of colored lights.

The Reactions of the Larvae to Light, Heat and Gravity

Diffuse daylight. As described by Grave (1930), the larvae are intensely photopositive during the first three or four hours after their release from the ovicells and then become photonegative. The writer found the photonegative reaction to be somewhat more intense than that described by Grave (1930), who concluded that "it might be overlooked" because of its gradual onset. By 6–8 hours both species of larvae were always definitely photonegative and this reaction was intensified by placing them in sea water diluted by 50 per cent. Furthermore, they changed their reaction from positive to negative immediately after they were placed in sea water buffered to a pH of 9.6 (borate buffer) or diluted by 50 per cent, even when the latter was made hypertonic by the addition of sucrose. In sea water containing 2.5 mg. of copper chloride per liter the majority of the larvae, still photopositive 30

minutes after exposure, did not swim towards the light when the dish was reversed as they normally do. When a concentration of 5 mg. copper per liter was used, they became photonegative within 30 minutes. Even 0.5 mg. per liter reduced the intensity of the photopositive reactions. Since these peculiarities were not observed when the organisms were ejected from a pipette into sea water, mechanical force can hardly be the cause of this reaction. The absence of photic responses in mixtures of sea water and magnesium chloride was mentioned in a previous paper (Lynch, 1949).

Reactions to blue and red light. When a prism spectrum was used to illuminate vials placed horizontally in a black box about 16 feet from a 500 watt bulb, the larvae aggregated densely in the yellow, orange and red regions during their photopositive phase but afterwards congregated in large numbers (80–90 per cent) in the dark region beyond the violet when only the middle portion of the vials was illuminated, or in the violet region when the whole tube was exposed. Apparently this behavior was merely a response to heat, which the larvae tried to avoid, or to light intensity rather than to color.

Since the above method was unsatisfactory, the remaining experiments were carried out with blue and orange-red Eastman Kodak Wratten filters, numbers 76 and 72 respectively. Number 76 transmits a wavelength of approximately 4200–4800 Å and number 72 a wavelength of 5800–6600 Å. These filters have nearly equal relative energy transmission when illuminated with a 400 watt bulb according to Hecht (1921). One end of the vials was illuminated by blue light and the other by red. In the center there were two dark bands caused by the opaque paper of the edges of the two filters where they touched each other. More specimens attached in these dark regions than in either the blue or the red end; these, of course, were not counted in the total number that responded to the colored lights. Of the 292 larvae used in 5 trials 67 per cent attached in the red end. Actually, due to errors in counting large numbers of larvae often attached one on top of the other, the percentage was probably larger than this. In four of the trials there were 1.5 to 2 times as many in the red end. The standard error of the proportion was obtained from the formula, $S.E._p = \sqrt{pq/N}$, for calculating the significance of the results. If it is assumed that the null hypothesis holds in this case, the expected percentages in each end would be 50 per cent (.50), and both p and q would equal .5 each. Thus the $S.E._p = \sqrt{(.5 \times .5)/292} = .0291$. Since an excess of .17 (67 per cent minus 50 per cent) over that postulated by the null hypothesis is at least five times the $S.E._p$, the results would fall easily within the range of "very significant." In these experiments *B. flabellata* showed as much uniformity of response as *B. turrita*, despite the absence of eye-spots on the former. Visscher (1927) obtained somewhat similar results with colored tiles, for more Bryozoa attached to red test panels than to green, black or yellow ones, and no settings occurred on the white ones. (Cf. also Edmondson and Ingram, 1939.)

Darkness. As Grave (1930) had observed, darkness delays fixation and favors attachment to the surface of the water. The writer found the larvae of *B. turrita* to be almost universally active at the end of 24 hours, when kept in complete darkness in a microscope case, and the majority continued to swim for three or four days (the normal duration of the natatory period of most larvae of this species does not exceed 24 hours). Specimens kept in bottles remained active longer than those

in uncovered syracuse dishes. Since the sea water in the latter became more concentrated by evaporation, the increased salinity hastened metamorphosis. (Cf. Lynch, 1947). By six days the attached organisms had elongated considerably (maximum length, 1.45 mm.), giving the surface of the water a fuzzy appearance; this was caused by a great abundance of transparent material, much of it in the form of four, symmetrically placed stolons for attachment to the surface film and the remainder organized into a club-shaped structure joined to the stolons and containing in its center an opaque spherical mass that closely resembled the unmetamorphosed larva. The eye-spots were generally visible either in the opaque mass or at a short distance from it in the transparent parts. Apparently development ceased after elongation, for polypides were never observed.

Effects of temperature. Heating the medium to 30° C. accelerated metamorphosis, and raising the temperature to 32–35° C. caused cytolysis; both effects were more pronounced in *B. turrita* than in *B. flabellata.*[2] Many of the former, mere rings of ciliated tissue without material in their centers, were often observed swimming slowly in test tubes exposed to light from a 500 watt incandescent bulb. Enormous amounts of adhesive material always surrounded the larvae after extrusion of the hold fast, but rigid attachment failed to occur. Expansion, especially by elongation along the apico-basal axis, followed exposure to heat. Sausage-shaped streamers of tissue from the pallial furrow, similar to those produced by exposing the larvae to sea water containing an excess of magnesium chloride (Lynch, 1949), were extruded by the larvae of *B. flabellata* from their apical ends, which always looked larger than normal. Development was poor or totally lacking in both species, even when cytolysis did not occur. Marcus (1926) briefly mentioned the accelerating effect of heat on bryozoan larvae.

Geotropism. When diffuse daylight enters a test tube of sea water horizontally, the larvae become fixed at various places along the side farthest from the window, although attachment to the surface is also very common. Under experimenal conditions the larvae generally fell to the bottom just after immersion in a new medium, especially if it contained an excess of various salts. When sea water was mixed with equal parts of normal solutions of sodium, potassium, magnesium or calcium chlorides, the larvae did not swim to the surface again, apparently because ciliary action was too feeble. In mixtures of 80 cc. sea water per 20 cc. of normal calcium chloride, however, vigorous swimming movements were maintained. Heating the medium to 30° C., keeping the larvae in darkness or immersing them in 80 cc. sea water per 20 cc. normal sodium chloride favored attachment to the surface. This concentration of sodium chloride, however, affected the two species somewhat differently. Since *B. flabellata* metamorphosed almost immediately in this mixture most of the larvae did not recover sufficiently from their initial "fright-reaction" to reach the surface, and floating larvae were not often observed; *B. turrita* re-

[2] Experiments on *B. neritina* had shown that a reduction of temperature from 23° C. to 7° C. caused all the larvae to become geopositive and lengthened the natatory phase by 2–3 hours (Lynch, 1947, p. 128). Since the writer was not interested in lengthening the larval phase of the Woods Hole species, similar experiments were not repeated. It cannot be assumed, however, that the results would have been similar, since Barrois (1879) reported that a bowl-full of Serialaria (Ctenostomata) that invariably attached at night, was placed in ice and maintained at a temperature near zero all night. Contrary to his expectations, setting took place in a normal manner.

mained active longer and many were at the surface when fixation took place. During the photonegative phase the larvae of *B. flabellata* swimming in normal sea water could be made to move downward by placing the light source above them or upward by illuminating them from below. (Cf. also Grave, 1930.) It seems likely, therefore, that in this species the positive geotropism that occurs in nature towards the end of the matatory period is brought about both by light and by a reduction in ciliary movement. In this respect the species at Woods Hole differ considerably from *B. neritina*, for geotropism in the latter is apparently independent of phototropic responses.

Discussion

The experiments just described and those presented in former papers (Lynch, 1947, 1949) show that heat, light, salinity and the relative proportions of ions in sea water can profoundly affect the natatory period of *Bugula* larvae and the subsequent growth of zooids. From an ecological standpoint, environmental fluctuations that affect the setting of larvae are of paramount importance. Hutchins (1945), having observed that adult species of Bryozoa grew quite well after being transplanted from their natural habitat to one where they were either rare or totally absent, concluded that "in all probability the critical tolerances of environmental variations are those of the larvae, particularly during metamorphosis when they may be supposed to be minimal." An interesting problem is posed by the peculiar distribution at Woods Hole of the two species of Bryozoa under discussion. Grave (1930) stated that *B. turrita* is found in Vineyard Sound, but not in the Eel Pond, whereas *B. flabellata* is abundant in the Eel Pond, but is not ordinarily found outside it, even though the two bodies of water are less than 300 feet apart and communicate freely with one another.[3] Although the adults when transferred from one region to the other on a raft may live for a few months or a year, all efforts to establish new colonies by the transplanted species have so far met with failure (Grave, 1930). A similar peculiar distribution of *Teredo navalis* and of certain hydroids can also be observed. What explanation can be given for these facts? That the larvae are extremely sensitive to the ions present in sea water is evident; whether they can complete metamorphosis and attain normal growth depends upon a very delicate balance of the chemical constituents of their environment. There are, however, so many variables affecting larval behavior that it is extremely difficult to isolate specific ones as causative agents of ecological distribution. Conceivably a particular species thrives best under conditions that enable the organism to terminate larval life after an optimum swimming-time, since larvae that are induced to swim long beyond the normal time of setting rarely develop or form zooids comparable to the controls in size or differentiation. Species such as *B. neritina* with an extremely short natatory period under laboratory conditions seem to be more adversely affected than those whose larval life is of longer duration. It may be that stored nutritive material essential for the formation of zooids is exhausted by prolonged swimming.

The behavior of the two species of *Bugula* from Woods Hole shows certain marked similarities to that of *B. neritina* (from the Beaufort region) and a few

[3] He referred, of course, to the natural habitat of the two species. Actually both species were found by the writer growing on a raft at the entrance to the Eel Pond (August, 1949).

striking contrasts. Their reactions to excesses of various metals are nearly identical (Lynch, 1947, 1949). (Copper, however, was not tried on *B. neritina*.) But there are significant differences in their responses to light and gravity. The larvae of *B. neritina* never have a photonegative phase at any time under laboratory conditions, although there is some evidence that they become indifferent to light just before setting. In this respect they resemble the European variety of *B. flabellata* described by Nitsche (1870). Furthermore, they almost universally remain near the surface, and up and down movements in a vial occur but rarely. The larvae of both species from Woods Hole, however, swim vertically along the side of the container during their photonegative phase as readily as they do horizontally during the transitional period when their phototropic responses are beginning to reverse.

The marked similarity in both phototropic and geotactic behavior of the two species from Woods Hole and the contrast that exists between the behavior of these species and that of *B. neritina* would suggest the possibility that environmental conditions in the two regions might be partially responsible for differences in behavior. Both the extreme brevity of the natatory phase of the Beaufort species and the failure of the larvae to become geopositive at any time under laboratory conditions would seem to be influenced at first sight by two obvious differences in environment, a higher temperature and a brackish condition of the water. This hypothesis is based on the experimental evidence that extreme variability of the duration of the natatory period of a given species can be brought about by altering the ionic balance of the medium, by changing the salinity or by varying the temperature. By altering these factors the geotropic behavior of the larvae can also be changed. Since the abundance of oysters in the brackish waters of Beaufort would suggest the probability that the copper content of this region might be greater than it is at Woods Hole, it would not be unlikely that this ion, capable of hastening the onset of metamorphosis in several sessile organisms, might be largely responsible for the extreme brevity of the larval stage of *B. neritina*.[4] The hypothesis that a greater concentration of copper in the Beaufort sea water and the higher temperature prevailing in that region might play a role in causing contrasts in behavior of the northern and southern species is not illogical, since the natant phase of *B. flabellata* can be shortened appreciably either by adding $CuCl_2$ to sea water or by raising the temperature of the medium. Furthermore, warming the sea water to 30° C. favors surface attachment of *B. flabellata*, whereas cooling the medium causes the larvae of *B. neritina* to become geopositive and prolongs their larval stage; thus, either species may be made to react like the other in this respect. Apparently the pelagic habits of *B.*

[4] In brackish waters, according to Prytherch (1934), the copper content may reach a concentration of 0.1–0.6 mg. per liter during low tide, whereas it rarely exceeds 0.02 mg./liter in the sea (Galstoff, 1943). Likewise the relative proportions of sodium, magnesium and calcium in brackish waters differ considerably from the distribution of these ions in the ocean, since the order of concentration of these ions approaches that of fresh water (calcium, magnesium and sodium). (Cf. Clarke, 1924.) Recently (after this paper had been prepared in its present form) Glaser and Anslow (1949) gave the copper content of Woods Hole sea water (spectroscopically determined) as $2.50 \pm \times 10^{-7}$ M Cu. They found that a sample of Beaufort sea water had a value as high as 1×10^{-6} M Cu; they noted, however, that the latter may have been contaminated (p. 127 and 128).

neritina are correlated with their brief natatory period (Lynch, 1947);[5] likewise the much more frequent occurrence of geopositive settings of *B. flabellata* under normal conditions seems to be related to their naturally longer larval phase, for the number on the bottom of a vessel begins to increase after the larvae have been active for several hours.

Nevertheless, even though the behavior (except phototropism) of one species can be duplicated almost exactly in the other by altering the environment, neither differences in temperature *alone* nor in the content of the sea water can account for contrasts in the behavior of the northern and southern species. The monthly mean temperatures during July and August at Woods Hole and at Beaufort differ by only six or seven degrees, according to McDougall (1943), and experiments performed at Beaufort by the writer showed no marked change in the behavior of *B. neritina* when the temperature was reduced to 21° C. (the monthly mean for Woods Hole), although a more drastic reduction of temperature did reverse the geotropism of the larvae and prolong their free-swimming phase. Likewise, when larvae of *B. flabellata* were immersed in sea water taken at low tide from the Beaufort region and shipped to Woods Hole, their behavior was like that of the controls. No shortening of the larval phase was observed. (The experimental sea water had a pH of 7.5 when used.) Logically, negative results were partially predictable, since previous experiments had shown that a concentration of copper chloride as low as 0.5 mg per liter of sea water (about maximum for brackish waters) had no appreciable effect in shortening the natatory period, although higher concentrations were effective.[6] Is it then a mere coincidence that the southern species with its short natant phase lives in an environment where two factors, a higher temperature and a presumably (?) greater concentration of copper, are both present and either of these can accelerate the onset of metamorphosis? There are two possibilities. On the one hand, since experimental modifications of temperature that proved to be effective in lengthening or shortening the larval phase were more drastic than those actually prevailing in nature and since the same was true of the copper content, conceivably slighter changes of both factors combined might be as effective as more extreme alterations of each one separately. It should be noted that, since the temperature of the Beaufort sea water was not raised to the degree ordinarily prevailing in that region, the environment of the southern species was only partially duplicated on the northern one. On the other hand, since there are generic and specific as well as individual differences in the natatory phase of bryozoan larvae, it seems more tenable to assume that species genetically determined to have a short larval period can thrive only in an environment where ions, presumably requisite for setting, can be rapidly absorbed. Conceivably, there may be specific differences in the ion-absorbing ability of larvae. This assumption, however, offers no explanation for the fact that the larval phase of the majority of a given species may end at two hours on one day and at ten hours on another.

[5] By using two sets of data that appeared to have the least positive correlation, the coefficient of correlation between the number of larvae of *B. neritina* that were kept active for four hours by a reduction of salinity and the number that became geopositive at the time of setting was found to be + .53 and + .57.

[6] It should be noted that concentrations of $CuCl_2$ as high as 1.25 mg. per liter had no effect; Prytherch (1934) found that in his experiments virtually all the copper was precipitated when less than .5 mg./liter was used.

(See Table I, p. 30, Lynch, 1949).[7] Before the relative effects of environment can be evaluated it is necessary to know whether there are significant differences in the length of the natatory period of the same species in different localities. Since Edmondson and Ingram (1939) reported that larvae of the Hawaiian *B. neritina* attach at night as readily as during the day, it may be that these organisms have a much longer natant phase than the ones studied at Beaufort. The latter (laboratory conditions) were always released a short time after exposure of the parental colonies to light, and active unattached ones could rarely, if ever, be found after noon. Caution must be observed, however, in making such an assumption, for it is unwise to conclude that the natatory period of larvae under natural conditions is as brief as it is in the laboratory. Indeed, the vertical distribution of adults indicates that in nature these larvae are probably active longer than an hour or two (maximum time in the laboratory) and undergo sufficient activity to make most of them geopositive. It would be extremely valuable to have definite information regarding the behavior of *B. neritina* on the California coast. There is also need for further research on the interaction of factors capable of accelerating or retarding metamorphosis. Some may have antagonistic effects; others may act synergistically.

The writer is indebted to Professors J. H. Bodine and H. W. Stunkard for reading the manuscript and to Dr. M. D. Rogick for information regarding the taxonomy of the genus *Bugula*.

Summary

1. The larvae of both *B. flabellata* and *B. turrita* are photopositive in diffuse light during the first 3–4 hours after release from the ovicells and then become photonegative. They became photonegative immediately, however, when they were placed in sea water buffered to a pH of 9.6 or diluted by 50 per cent; sea water containing copper chloride either reduced the intensity of the photopositive phase or caused a reversal of phototropism depending on the concentrations that were used. These organisms became indifferent to light in mixtures of 80 cc. sea water per 20 cc. of either normal calcium chloride or magnesium chloride.

2. Larger numbers of larvae attached in the red end of a test tube illuminated by red and blue light passing through Wratten filters than in the opposite end.

3. Heating sea water to 30° C. hastened metamorphosis and favored surface attachment, but development was poor or entirely lacking at this temperature. Darkness delayed metamorphosis and also caused attachment to the surface film;

[7] Barrois (1879) stated that in the laboratories at Roscoff the same species of Bryozoa generally showed extreme variations of the natatory period on different days and that cases of this strange phenomenon could be found in all groups but was especially striking in *Pedicellina* and *Cyphonautes*. In some cases (*Flustrella hispida*, especially) he found it impossible to obtain a single fixation during a period of many weeks, even though the larvae were very abundant. At other times, under apparently identical conditions, fixations took place in large numbers. He noted that the incapacity of larvae to fix themselves might persist for a long time or cease suddenly. At times settings occurred in various parts of the bowls; at other times they took place *en masse* at certain points. But these anomalies were entirely absent in bowls prepared at the same time and place and under identical conditions. Harmer (1922, p. 513), likewise, noted the difficulty of persuading larvae to attach under laboratory conditions and stated that it could be surmounted by placing adult colonies in a vessel closed with fine muslin and left attached to a buoy or placed in a deep tide-pool. Evidently environment is extremely important.

development ceased after a fair amount of growth and a slight degree of differentiation.

4. In mixtures of equal amounts of sea water and normal solutions of sodium, potassium, magnesium and calcium chlorides the larvae became geopositive on entering the medium and remained so during the experiments.

5. Some ecological problems of the distribution of three species of *Bugula* are discussed and tentative suggestions for their solution are offered.

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THE RESISTANCE OF SCIARA (DIPTERA) TO THE MUTAGENIC EFFECTS OF IRRADIATION

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On the basis of the irradiation studies made on Sciara to date, a condition has been noted which is of general interest with reference to the mode of action of x-rays on the hereditary material, namely, an apparent resistance to the mutagenic effects of irradiation. Whereas gross and minute chromosome rearrangements are induced in treated germ cells, visible mutations appear at a negligible frequency.

Sciara is very unusual in this respect. In other organisms, including Drosophila, maize, and Neurospora, x-rays are found to induce both mutations and chromosome breaks at frequencies proportional to the dosage. During the past twenty years at least eight investigators have independently looked for visible mutations among the progeny of irradiated Sciara; altogether only twenty-four or twenty-six mutant characters have been obtained in the several species treated. Unfortunately, the irradiation data are not tabulated in such a way that an estimate can be made of the number of germ cells exposed or the total progeny examined. But certain of the studies were extensive. Both male and female germ cells were treated at various times in the developmental cycle and dosages from 3000 to 30,000 r. applied.

In view of the low mutation rate obtained repeatedly (see Metz, 1938), the chromosomes of Sciara were believed to be resistant to irradiation. The first clue to the contrary was the discovery of a reciprocal translocation in the salivary gland nuclei of larvae taken from cultures of the "Stop" mutant (Crouse and Smith-Stocking, 1938). The salivary gland chromosomes were then utilized in a cytological analysis of F_1 larvae derived from irradiated sperm or oocytes (Metz and Boche, 1939); following exposure of sperm to 5000 r., approximately 25 per cent of the F_1 showed gross chromosomal rearrangements. This unexpected induction of chromosome aberrations in Sciara was confirmed in subsequent experiments.

The experiments reported in this paper were performed in connection with cyto-genetic studies on the unusual behavior of the sex chromosome of Sciara. The data bear on the problem at hand, however, and will therefore be discussed in this relation.

Preliminary data on dominant lethal induction are in line with the rearrangement studies and provide further evidence that the chromosomes of Sciara are sensitive to irradiation. In one experiment designed to pick up X-translocations in *S. coprophila*, nine females were bred singly to adult males which had been x-rayed at 4000 r. The nine females yielded 108 total offspring, while nine control females from the same stock (isogenic) produced 723 total offspring. The exact probability (as measured by chi-square) of this difference is 0.0004. On the basis

of this small but very carefully conducted experiment, only 15 per cent emergence was obtained, a dominant lethal value which is practically identical to that measured by Demerec and Fano (1944) in Drosophila sperm x-rayed at 4000 r. It is of interest to note that in the Sciara experiment cited, nine of the 108 survivors (8 per cent) were heterozygous for X-translocations but none transmitted sex-linked visible mutations.

If dominant lethals are regarded as the result of certain types of chromosomal aberrations (see Pontecorvo, 1942), Sciara and Drosophila chromosomes respond to irradiation in a similar manner, and the physiological result (i.e., the lethal phenotype) is the same in both genera. It is with respect to less drastic physiological changes—namely, hereditary alterations classified as "visibles"—that the two genera differ.

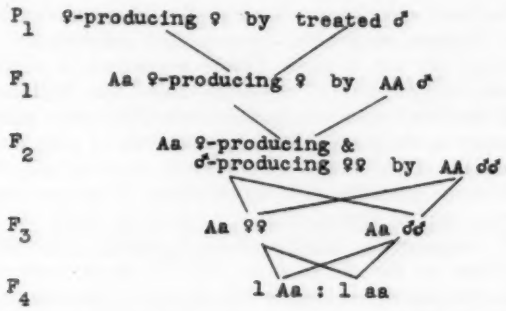
Several factors may account for the low visible mutation rate in Sciara.

(1) The external appearance of this fly (bristle pattern, pigmentation, etc.) is such that only the most conspicuous visible changes are likely to be detected.

(2) There is a distinct difference between the autosomal and the sex-linked mutations, which suggests that the induced mutation rate in this genus is considerably greater than the detected mutation rate. In *S. coprophila,* the species which has been most thoroughly worked, nine autosomal and five sex-linked mutations have been recovered. Of the autosomal group, seven are dominant and two are recessive; among the sex-linked factors, on the other hand, four are recessive and only one is dominant. The exact probability (as measured by chi-square) of this difference is 0.126. In material such as Drosophila, Habrobracon and maize, the dominant mutations constitute a very small percentage of the total number of visible mutations. The relatively high proportion of dominants in Sciara has been interpreted as evidence that this genus is unique in its response to irradiation (Metz, 1938). Such an interpretation is probably not valid, since, as noted above, the dominants constitute a majority only in the case of the autosomal factors. Most likely the discrepancy in the autosomal mutations has a relatively simple explanation, namely the extraordinarily tedious and inefficient technique available for the detection of autosomal recessives in this genus.

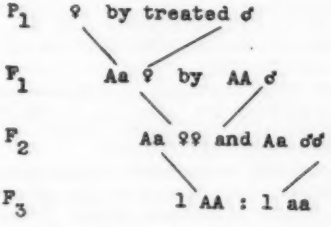
The mode of inheritance and the mechanism of sex determination in Sciara make it difficult to pick up autosomal recessive mutations. *S. coprophila* is monogenic. This means that the females produce either sons or daughters but not both; consequently, an F_1 female derived from an irradiated sperm will yield a family of sons or a family of daughters. If she is a male-producer and heterozygous for an induced sex-linked recessive, half of her sons should show the mutant character. Sex-linked recessives, therefore, can be fairly readily detected in Sciara in the F_2 generation. Autosomal recessives, on the other hand, are practically impossible to pick up because of the monogenic condition described above and because Sciara males transmit only the genes they inherit from their mother; the paternally derived chromosomes are eliminated at the first spermatocyte division. In searching for autosomal recessives, the best procedure is to take the mutated gene (a mutation induced in treated sperm), through the female germ line according to the scheme outlined below.

In order to detect the recessive mutation, a, four successive generations of flies (approximately four months at 70° F.) have to be produced as outlined; and then



the probability that the mutation will be detected in the F_4 is less than $1/16$. The calculation, of course, does not include I, the probability of induction of the mutation. In the sex-linked mutant strains of *S. coprophila,* male- and female-producers are phenotypically distinguishable. If one of these mutant strains is used in the experiment diagrammed above, the chance of selecting a ♀-producing female and a ♂-producing male of genotype Aa in the F_2 is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$ (F_2 females are AA or Aa). Then the chance of selecting a female and a male of genotype Aa in the F_3 is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$, making the final probability $I \times \frac{1}{4} \times \frac{1}{4}$. Thus, the odds against detection of an autosomal recessive in *S. coprophila* are at least 15:1.

In digenic species like *S. reynoldsi* (the females produce both sons and daughters), autosomal recessives can be detected in the F_3 generation according to the following scheme:



In this case the chance of selecting an F_2 male and female of genotype Aa is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$, making the odds against detection of the mutation only 3:1 instead of 15:1.

The evidence presented so far is consistent with the view that the detected mutation rate in Sciara is considerably less than the induced rate. Even so, the induced rate appears to be less than that observed in Drosophila or Habrobracon; for, in the case of the sex chromosome of *S. coprophila,* where recessives can be picked up in the F_2, only five factors (four recessive and one dominant) have been found.

This dearth of mutants is particularly significant in view of the fact that over a period of several years the author has tested a large number of irradiated sperm (approximately 500) and oocytes (approximately 100) of *S. coprophila* in a search for reciprocal translocations which involve the X chromosome (Crouse, 1943 and

unpublished). In these experiments, breeding tests were carried to the F_2 generation or beyond; fourteen analyzable (genetic and cytological) X-translocations have been recovered but not a single visible sex-linked or autosomal mutation. Similar experiments conducted on *S. reynoldsi* yielded four X-translocations and no mutations among irradiated sperm (approximately 200 sperm tested).

(3) The presence in the germ cells of *S. coprophila* of large amounts of heterochromatin, in the form of the limited chromosomes, may actually retard the mutation rate. There are no published data on the effects of heterochromatin on general mutability either in corn or Drosophila—two forms in which the B-type chromosomes and the Y, respectively, might well be exploited. Unpublished studies of Miss Jean Kerschner, on the effects of an extra Y chromosome on the x-ray induced visible and sex-linked recessive lethal rates in *D. melanogaster* males, indicate that the extra Y brings about a significant decrease in these mutation rates.

(4) Finally, it is conceivable that, as compared to Drosophila or Habrobracon, the biochemical pathways which are available in Sciara result in a more restricted range of phenotypic variability.

Summary

Several facts have been discussed which may contribute to the apparent resistance of Sciara to the mutagenic effects of irradiation. New data are presented which (1) support the view that the visible mutation rate induced in this genus is lower than that found in Drosophila or Habrobracon; and (2) reveal a high dominant lethal mutation rate in this genus.

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RECOVERY FROM ULTRA-VIOLET LIGHT-INDUCED DELAY IN CLEAVAGE OF ARBACIA EGGS BY IRRADIATION WITH VISIBLE LIGHT [1]

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Kelner has reported that exposure of *Streptomyces griseus* conidia to intense visible light (V.) after inactivation with ultra-violet light (U.V.) enabled these conidia to grow (Kelner, 1949). Dulbecco obtained similar results when bacteria containing U.V.-inactivated bacteriophage were given visible light (Dulbecco, 1949). The experiments to be described show that a similar phenomenon occurs when *Arbacia punctulata* eggs fertilized with U.V.-treated sperm are exposed to intense visible light.

Methods

The U.V. source was a Westinghouse Sterilamp W.L. 782, 80 per cent of whose radiation was at $\lambda = 253.7$ mμ. The visible light was produced by two 1000-watt tungsten filament lamps, the light from each being focused through a round, water-filled, one-liter pyrex flask onto a white tile. An infra-red filter was placed 5 inches from the illuminated spot which was 2 inches in diameter. A fine-grained ground-glass screen was placed 2 inches above the tile in order to diffuse the filament images. The U.V. intensity, measured with a Hanovia Ultraviolet meter, was 52 micro-watts per cm^2 at the position where the biological material was irradiated. The visible light intensity on the white tile, as measured with a Leeds-Northrop Macbeth Illuminometer, was approximately 28,000 foot candles. The rise in temperature of the irradiated suspension, as measured with a thermometer immediately after irradiation, was less than 0.2° C. The heat generated by the lamps was dissipated by circulation of air with an electric fan. With windows and doors of the laboratory kept open, there was no appreciable rise in the temperature of the room.

Sperm suspensions were prepared by dispersing 2 drops of "dry sperm" in 250 ml of sea water ("0.4 per cent sperm"). Egg suspensions contained 5–10,000 eggs/ml. For irradiation with either V. or U.V., 2 ml of either suspension was pipetted into a pyrex petri dish (without cover) 2 inches in diameter, which formed a layer about 1 mm high. To fertilize eggs, 0.2 ml of the sperm suspension was added to the 2 ml egg suspension with agitation.

The time for cleavage was obtained by counting the number of cleaved and uncleaved eggs, the total being 50 to 100 eggs each time. After determining the point at which 50 per cent of the eggs cleaved, they were again counted to determine total cleavage. Data were discarded unless the controls showed a final cleavage of 95 to 100 per cent.

[1] Supported by a grant from the U. S. Public Health Service.

Temperatures were recorded but not controlled. In all experiments, temperature fluctuations were less than 1° C. in the course of the experiment.

Results

1. *Time for cleavage as a function of U.V. dose*

Sperm were irradiated for periods from 0.5 to 4.0 minutes and unirradiated eggs fertilized 8 to 10 minutes later. Since at the higher doses cleavage did not reach 50 per cent, comparison was made at the 10 per cent cleavage point. The following table gives the results obtained:

(24° C.)

Exposure time (min.)	0	0.5	1.0	2.0	3.0	4.0
Time to 10 per cent cleavage (min.)	47	88	113	157	214	235

Eggs were treated with U.V. and then fertilized by normal sperm with results as shown below:

(24° C.)

Exposure time (min.)	0	0.5	1.0	2.0	3.0	4.0
Time to 50 per cent cleavage (min.)	47	49	58	62	68	72

2. *Growth of the U.V. effect*

Sperm and egg suspensions were irradiated with U.V. and allowed to stand for varying lengths of time before fertilization. The sperm was irradiated for 0.5 minutes, the eggs for 5.0 minutes. The following results were obtained when irradiated sperm was used to fertilize normal eggs and irradiated eggs were fertilized with normal sperm:

Time for 50 per cent cleavage (25° C.)

T	2	10	20	30	40	50	60	80	c	c′
Irradiated sperm	98	100	98	104	107	110	120	124	44	47
Irradiated eggs	69	71	69	68	68	68	73	70	47	47

T = time in minutes from irradiation to fertilization
c = non-irradiated eggs and sperm. Fertilization immediately after preparation of suspensions.
c′ = non-irradiated eggs and sperm. Fertilization 75 minutes after preparation of suspensions.

There was an appreciable growth of the cleavage delay factors in the irradiated sperm, but there was neither growth nor decay of these factors in the egg.

3. *Irradiation with ultraviolet and visible light before fertilization*

To determine whether V. given to either gamete before or after U.V. irradiation altered the U.V.-induced cleavage delay, eggs and sperm were separately irradiated. The irradiated sperm were used to fertilize normal eggs and irradiated eggs fertilized with normal sperm. The results are given below:

Time for 50 per cent cleavage (25° C.)

	Exposure time V.	Exposure time U.V.	Control	U.V.	V.	V. then U.V.	U.V. then V.
Sperm irradiated	3′	10″	46	70	46	69	68
	3′	30″	46	117	—	114	109
	3′	2′ [2]	41	139	—	135	133
Eggs irradiated	3′	2′	48	72	47	72	74

Visible light given to either gamete did not change the cleavage time. Visible light given before or after U.V. did not significantly shorten or prolong the U.V.-induced delay in cleavage.

4. *Visible light given to the zygote*

When visible light (3 minute exposure) was given to eggs fertilized with untreated sperm at various times after fertilization, the time for cleavage was not significantly affected. The following shows the results obtained:

			V. − 3′				*t* − 27° C.
I	2	5	10	15	20	30	c
T	41	41	40	43	42	44	44

I = Time of irradiation with visible light in minutes after fertilization. I is measured to the time of beginning of irradiation.
T = Time in minutes for 50 per cent cleavage.
c = Control not exposed to light.

However, when the zygote was exposed to visible light after fertilization with U.V.-treated sperm, there was marked reduction in the time for cleavage. The following are results from three runs at different times to indicate the degree of reproducibility. In all cases the eggs were fertilized within 10 minutes after U.V. irradiation of the sperm. Repetition of these experiments several times gave essentially the same results:

				U.V. − 0.5′, V. − 3′							
I (26° C.)	1.5	5	9	11	15	20	30	40	60	c′	c
T	60	58	56	54	69	81	91	94	98	97	44
(27° C.)	2	6	10	35	50					c′	c
T	65	60	56	85	90					97	43
I (25° C.)			10	50	60	70	80			c′	c
T			59	87	90	93	95			96	44

I, T, and c as above.
c′ = eggs fertilized with U.V. treated sperm but not exposed to visible light.

The delay in cleavage (inactivation) is considerably shortened when exposure to V. begins as early as 1.5 minutes after fertilization. This shortening of the cleavage-delay time will be referred to as photoreactivation. Maximum efficiency in photoreactivation was observed when exposure to visible light began at 9 to 11 minutes after fertilization. Detectible photoreactivation was observed as late as 40 to 50 minutes after fertilization.

[2] In this series, values are for time to 5 per cent cleavage.

Neither U.V. inactivation nor photoreactivation had an appreciable effect on the time for the second cleavage. Below are given the observed times between first and second cleavages after U.V.–0.5′ and V.–3′ at the times indicated:

I	10	50	60	70	80	c′	c
T[3]	26	30	32	32	30	32	26

I, T, c′, c as above.

It was noted in some experiments that c′, and the samples receiving V. at times later than 20 minutes after fertilization, had a considerable percentage of the eggs with a 3 to 4-cell first cleavage. This occurred at about the same time that the remaining cells showed the usual 2-cell cleavage. These 3 to 4-cell first cleavages were absent or rare in c and in eggs receiving V. at 10 to 20 minutes after fertilization. In these cases when allowance was made for the percentage of 3 to 4-cell zygotes by first cleavage, and the time determined for 2-cell zygotes to reach the 4-cell stage, the results were essentially the same as those given above. The factors responsible for the production of 3 to 4-cell first cleavages were not investigated.

5. *Quantity of light for maximum photoreactivation*

Following irradiation of sperm with U.V. for 0.5′, eggs were fertilized and the fertilized eggs exposed to the visible light, exposure beginning at 10′ after fertilization in all cases. The exposure time was varied with the results shown below:

				t – 24° C.				
E	10	30	60	120	180	300	c′	c
T	87	77	75	78	63	66	99	58

E = exposure time in seconds.
T, c′, c as above.

The data show that exposure for periods longer than 3 minutes did not produce greater photoreactivation.

6. *Photoreactivation in nucleated white halves of Arbacia eggs*

Eggs were centrifuged at approximately 10,000g for 10 minutes and the nucleated white halves and non-nucleated red halves collected separately. These were then fertilized with U.V. treated sperm and the zygotes exposed to visible radiation with the following results for the white halves:

			U.V. – 0.5′, V. – 3′			t – 26° C.	
I	10	20	30	50	65	c′	c
T	72	83	95	100	101	106	46

I, T, c′, c as above.

Photoreactivation was, therefore, possible in the non-pigmented half-eggs although the capacity for photoreactivation appears to be lower than in the whole egg. Since cleavage in the red halves was rare, the presence or absence of the capacity of these pigmented half-eggs for photoreactivation was not investigated.

[3] Time measured from 50 per cent first cleavage to 50 per cent second cleavage.

7. Photoreactivation efficiency of light of different wave-lengths

Wratten filters, 3″ × 3″, were mounted on the ground glass screen with the edges held by scotch tape, and oriented so that all of the beam passed through the filters. With filter No. 73, which transmitted at 560 to 620 mμ (maximum 570 mμ) and to some extent at 680 to 700 mμ and had a total transmission of 1.6 per cent, the following results were obtained:

	U.V. − 0.5′, V. − 8.0′				t − 24° C.	
I	10	20	60	c′	c	c″
T	80	94	107	106	50	57

I, T, c′, c as above.
c″ = full visible spectrum for 3′ at 10′ after fertilization.

Filter No. 76, which transmitted at 330 to 470 mμ (maximum at 440 mμ) and very little at 690 to 700 mμ with total transmission at all wavelengths of 0.1 per cent, gave the following results:

	U.V. − 0.5′, V. − 8.0′				t − 24° C.	
I	10	20	60	c′	c	c″
T	80	94	126	120	51	57

I, T, c′, c, c″ as above.

The same amount of photoreactivation was obtained with either filter. Since the total transmission of No. 76 was less than that of No. 73 by a factor of 16, the photoreactivation efficency of the band 330 to 470 mμ was much greater than light of wavelengths 560 to 620 mμ. The intensity of the filtered light was too low to obtain maximum photoreactivation in both cases, but this does not affect the validity of the above deduction.

8. Time for the "streak" stage

In addition to the time for 50 per cent cleavage, the time when the "streak" stage first appeared was also noted. In 6 experiments, this time varied from 13 to 18′, but in each case the time was the same, within one minute, for the non-irradiated controls and those which had received U.V.-treated sperm. Since the streak represents the development of the sperm aster, U.V.-irradiation apparently affects a later stage.

9. Treatment with chemical agents [4]

Streptomycin, in concentrations of 1 μg/ml to 10 μg/ml, had no effect on cleavage time of eggs fertilized with normal sperm. When sperm were given U.V. in the presence of streptomycin (1 mg/ml), there was no significant change in either U.V.-inactivation or photoreactivation.

Treatment of eggs with adenosine (20 μg/ml), prior to fertilization with U.V.-treated sperm, did not alter cleavage time, nor did it affect photoreactivation. Ad-

[4] We are indebted to the Lederle Laboratories for the folic acid and 4-amino-n-methyl folic acid, the Merck Laboratories for the streptomycin, and the Wellcome Research Laboratory for the 2,4-diamino-5-p chlorophenoxypyrimidine.

dition of adenosine to sperm immediately after U.V.-irradiation and fertilization 20′ later, also had no effect.

Eggs treated with folic acid at concentrations up to 10 μg/ml for 20′ before fertilization, or at 7′ after fertilization, with U.V.-irradiated sperm, showed no change in cleavage-delay time.

Eggs treated with 4-amino-n-methyl folic acid (10 μg/ml), 5′ after fertilization and for 20′ before fertilization, with U.V.-treated sperm, showed no effect on cleavage delay or photoreactivation.

Eggs were also treated with the purine antagonist 2-4-diamino-5-p-chlorophenoxypyrimidine (Falco and Hitchings, '49) for 30′ before fertilization with normal and with U.V. treated sperm. No effect was observed on the normal cleavage time, the U.V. inactivation or photoreactivation.

When U.V.-irradiated sperm were treated with riboflavin (20 μg/ml) or with riboflavin (20 μg/ml) plus adenosine (20 μg/ml) for 15′ before their use in fertilization, there was no effect on cleavage delay. Neither of these substances nor both given together affected normal cleavage time. When sperm treated with riboflavin were exposed to the visible light, they showed first a peculiar rapid vibratory motion and then complete cessation of motion within 10 minutes. Eggs could be fertilized with such sperm immediately after exposure to light. When eggs were thus fertilized, the time for cleavage was prolonged much beyond the usual U.V.-delay time. The cells attempting to cleave became very much elongated and twisted, but were only partially constricted. However, eggs treated with riboflavin for 20′ before fertilization with U.V.-irradiated sperm and not exposed to visible light, cleaved normally and showed no change in the cleavage delay time.

Discussion

Kelner (1949) and Dulbecco (1949) have both suggested that photoreactivation may be a general biological phenomenon. The experiments described here indicate its presence in an organism very different from either of those previously studied. It is interesting that in this respect the combination bacterium-phage is analogous to the combination egg-sperm. This analogy suggests that it would be desirable to determine whether inactivation of the phage could be obtained by U.V. irradiation of the bacterium prior to infection, and whether photoreactivation then follows the same course as in the case of U.V.-irradiated phage.

The present experiments show that there is a critical stage in the interaction of the sperm and egg when susceptibility to photoreactivation is at a maximum. The experiments on the time for the second cleavage also show that the cleavage-delaying conditions or agents are removed by the end of the first cleavage. The delay in cleavage is not due to inhibition or delay in the formation of the sperm aster. The effect must therefore be on some later stage. Once the cleavage constriction begins, the time for completion of cleavage is not significantly different in eggs receiving normal or U.V.-treated sperm within the dosage limits used in these experiments. The overall delay may therefore be attributed to a delay in the onset of prophase or to a prolongation of the prophase-metaphase time, or both. Since maximum sensitivity is at the time of onset of prophase, this appears to be the stage at which the U.V. inhibition becomes effective.

The agent or condition produced by U.V. remains unaltered in the unfertilized

egg but increases with time in the sperm, suggesting that stabilizing factors present in the egg are absent in the sperm, so that reactions initiated by U.V. absorption are much retarded or stopped in the egg but not in the sperm. The Arbacia egg contains a very considerable amount of ribonucleic acid in its cytoplasm (Schmidt, Hecht and Thannhauser, 1948 and Brachet, 1933). Consequently the relative inefficiency of U.V. irradiation of the egg may be taken as an indication that U.V.-alteration of ribonucleic acid of the cytoplasm is not involved and that the system affected is in the nucleus. The occurrence of photoreactivation in the non-pigmented half-egg indicates that the pigment is not involved in this reaction; and since most of the granules are removed, the latter also are probably not involved.

The relatively high efficiency of light of wavelengths in the region 360 to 460 mμ, suggests the possibility that intracellular substances with absorption bands in this region may somehow be related to the photoreactivation phenomenon. Riboflavin and folic acid both have absorption bands in this region (Warburg and Christian, 1938 and Stokstad, Hutchings and Subbarow, 1946). However, folic acid given to eggs or sperm does not alter the U.V.-inactivation. Also the folic acid inhibitor, 4-amino-n-methyl folic acid, does not prolong cleavage of the normally fertilized egg nor alter the U.V.-induced cleavage delay. The purine antagonist, 2-4-diamino-5-p chlorophenoxypyrimidine, also has no effect. No definite conclusion can be drawn from the failure to observe effects with any of these substances, since there is no proof that they penetrated the eggs or sperm. An exception may be made in the case of riboflavin since a marked effect was observed when the eggs or sperm were exposed to light. However, the effects observed with riboflavin-treated irradiated sperm seem to be unrelated to photoreactivation. Eggs treated with riboflavin, and U.V.-irradiated sperm similarly treated, show the usual cleavage delay time. The prolongation of cleavage, and abnormalities thereof produced by sperm irradiated with visible light in the presence of riboflavin, may be attributed to toxic photodecomposition products of riboflavin.

Conclusions

1. Irradiation of either sperm or egg with ultraviolet light will delay the time for the first cleavage division of the zygote formed by such gametes. Since irradiation of the egg is much less effective, the U.V.-inactivation is probably produced in the nucleus. The time of the formation of the sperm aster is not affected.

2. There is no decay or growth of the U.V. effect with time, after irradiation of the egg; but in the sperm there is definite growth.

3. Irradiation of either gamete with intense visible light, after U.V. irradiation, does not alter the cleavage delay.

4. Irradiation of the zygote with intense visible light reduces the cleavage-delay time. The zygote is most reactive at 10 minutes after fertilization, which is approximately the time of onset of prophase, although detectible response may be observed as late as 50 minutes after irradiation. The mitotic stage which is delayed and which responds most to the visible light is the onset of prophase.

5. Treatment with adenosine, streptomycin, folic acid, and 2-4-diamino-5-p chlorphenoxypyrimidine had no effect on either U.V.-inactivation or photoreactivation.

6. Treatment of the egg or zygote receiving U.V.-inactivated sperm with riboflavin, had no effect on the cleavage time. Irradiation of sperm in the presence of riboflavin with visible light, had a toxic effect on the sperm and on the zygote formed from such irradiated sperm.

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TRACHEAL FILLING IN SCIARA LARVAE

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Introduction

During insect development the gas-filled tracheae of a given instar become enclosed in larger, liquid-filled, coaxial tubes which are to form the tracheal system of the next instar. At molting the old tracheae are withdrawn and shed with the body cuticle. The new system then fills with gas, either immediately or within a short time, the liquid probably passing through the tracheal wall into the blood or tissues (Weismann, 1863; Palmén, 1877; Keilin, 1924, 1944; Davies, 1927; Wigglesworth, 1938; Keister, 1947, 1948). We shall call the process by which gas replaces the tracheal liquid "tracheal filling."

It is frequently assumed that the gas which appears in the new tracheae of insects with open tracheal systems is atmospheric air which has entered through the spiracles. This assumption is supported by the fact that in some insects the tracheae do not fill with gas unless the spiracles are exposed to free air. In other species, however, the tracheae can fill with gas even when the larva or embryo is submerged in water or surrounded by amniotic fluid, and many aquatic insects with closed systems normally fill their tracheae without apparent contact with an external source of gaseous gas (for literature see Keilin, 1924, and Sikes and Wigglesworth, 1931). It thus appears that in different insects there are at least two distinct mechanisms of tracheal filling, differing as to the source of the gas.

A number of explanations of tracheal filling have been proposed.

Weismann claimed that growth of tracheae (increase in diameter and number) continues after a change in permeability of the lining prevents entry of further liquid. The original liquid then retreats into the finer branches where it is absorbed, and gas enters from the outside or diffuses in from the tissues to occupy the increased tracheal volume. Major objections to this idea are the facts that, at least in Sciara, the tracheae have the same diameter when originally laid down as when they fill, and that no new branches are added near the time of filling (Keister, 1948).

Stadtman-Averfeld (1923) suggested that the violent body movements at molting expel the liquid from the tracheae, after which air enters through the spiracles.

Tillyard (1916), from the equivocal observation that tracheae collapsed when dragonfly larvae were put in KOH, proposed that the gas in the tracheae was CO_2 which diffused in from the hemolymph and displaced the liquid. Keilin (1924) pointed out several difficulties with this hypothesis. An additional objection is that the hydrostatic pressure in the tracheal liquid would have to be less than in the body liquids in order for gas evolution to be confined to the tracheae.

Filling has been attributed to reduction in body hydrostatic pressure at hatching (Davies) or after eclosion (Fraenkel, 1935). However, such reduction would not bring about the disappearance of the tracheal liquid unless the pressure had pre-

viously opposed some other force (e.g., osmotic) tending to withdraw the liquid into the body. Furthermore, it would not necessarily lead to the concurrent appearance of gas in the tracheae except in insects with spiracles open to the air. In closed tracheal systems, reduction in hydrostatic pressure might indeed bring about a preferential evolution of gas in the tracheal liquid (in the unlikely event that the tracheal liquid was higher in dissolved gases than the other body liquids) but would not account for the disappearance of the liquid itself. In any case, the facts that some embryos fill their tracheal systems before hatching, and that many insect eggs apparently have a low internal pressure (Sikes and Wigglesworth), argue against the idea that hydrostatic pressure has any major role in tracheal filling.

From ingenious experiments on the osmotic control of gas movement in the "tracheoles" of mosquito larvae, Wigglesworth (1930) postulated that increased tissue osmotic pressure due to muscular activity at molting could explain tracheal filling. Such a mechanism might conceivably lead to the replacement of tracheal liquid by outside air in insects with open systems, if the tracheal wall were semipermeable and the tracheal liquid markedly hypotonic to the body liquids as Wigglesworth postulated. However, neither of these conditions has been shown directly and unequivocally to occur, and indeed the latter seems rather improbable in view of Keilin's (1924) and Wigglesworth's (1939) opinion that the tracheal liquid is molting fluid, and Keister's demonstration that it originates by direct cytoplasmic transformation.

Wigglesworth later (1938) discovered that mosquito larvae hatched and kept completely under water for some days do not fill during the same stadium, even when returned to contact with air. From this and other considerations he concluded that the essential factor in normal filling was "secretion" of gas by the cells bounding the tracheae (these presumably being inactivated by long hypoxia in the submersion experiments). Gas secretion by hypothetical specialized cells or protoplasm was also invoked by Sadones (1895), Winterstein (1912), von Frankenburg (1915) in connection with this hydrostatic pressure theory, Pause (1918), and Akehurst (1922). A major objection to this proposal is the lack of any direct cytological, physiological and physical evidence of how secretion of gas might occur.

Keilin (1924) suggested that sudden absorption of tracheal liquid by the surrounding tissues (due to "imbibition or to a chemical reaction") would allow gas to diffuse from the tissues into the vacated space, provided the trachea could withstand a pressure of about an atmosphere. Bult (1939) similarly proposed imbibition as the explanation of the movements of tracheolar liquid and gas which Wigglesworth had attributed to osmotic forces. However, in neither Keilin's account nor in Bult's elaborate hypothesis is there any concrete evidence of the existence or nature of imbibitional changes.

An important characteristic of tracheal filling which must be accounted for by any proposed explanation is its great speed. It seems evident that at least the growth hypothesis of Weismann does not meet this requirement, and it has not been demonstrated that any of the other postulated mechanisms do.

The above review indicates that tracheal filling is far from being satisfactorily explained, and that it has not always been recognized that a process which might account for the appearance of gas in the tracheae would not necessarily explain the disappearance of liquid, or vice versa. The present investigation was undertaken in an attempt to clarify some of the problems outlined above.

Material and Methods

Living larvae of the mycetophilid fly *Sciara coprophila* Lintner were used usually at the beginning of the fourth stadium. The anatomy and development of the tracheal system, the process of molting and methods for raising larvae of known age have been described previously (Keister, 1948). Exposure to gases was carried out in brass cylinders each 22 mm. in diameter, 8 mm. high and with a wall 1 mm. thick. Each chamber had lateral inlet and outlet tubes and was cemented by one rim to an ordinary microscope slide. High humidity was maintained by lining the chamber wall with wet filter paper, and by bubbling the incoming gas through water. The larva was placed ventral side up in a minute droplet of water on a coverglass which was then inverted on the greased top rim of the chamber. Larvae so mounted could be observed for many hours, and at all magnifications. The work was done at $25 \pm 3°$ C.

For convenience in finding larvae in the proper stage, cultures were made up in petri dishes. Individuals about to molt are recognized by the fact that they stop locomoting and feeding and lie extended, usually with the anterior end raised, and make only slight and slow movements. Also, the tracheae are less distinct than in younger third instars due to the future fourth instar system enclosing them, and to the fact that usually much of the gas in the third instar system is replaced by liquid shortly before molting occurs. Further characteristic behavior usually involves: a period of axial back-and-forth rotation of the proventricular region of the gut; a period of slow, strong peristaltic waves of the body wall, alternately forward and backward; and finally, repeated bulging of the body just behind the head capsule. Soon thereafter the dorsal wall of the capsule and the cuticle just behind it split longitudinally, and the larva frees its anterior end and crawls forward out of the tubular exuviae, which are anchored to the substratum by viscid strands. Since the new tracheal system normally remains liquid-filled only for a short time after molting, larvae were mounted as quickly as possible after the splitting of the head capsule (usually within 1 minute). It was generally desirable to hasten the larva out of the cast by gentle prodding at the posterior end.

Observations

Normal tracheal filling

As described previously (Keister, 1948), visible gas is not present in the tracheae of newly-hatched (first stadium) Sciara larvae, and is found in only about one larva out of three even at the end of the stadium. When present, however, its extent is very constant. The actual filling process was never observed in spite of very numerous attempts. However, for reasons given in the earlier paper, it is considered unlikely that the gas enters by way of the posterior spiracles, which are the only pair present at this stage of development.

Second and third stadium larvae are less favorable for detailed study than fourth instars because of their smaller size. However, enough younger larvae were studied to show that tracheal filling after the first and second molts is essentially the same as described below.

Following the third molt, gas appears in 3 to 8 minutes and spreads throughout the (fourth instar) system in 1 to 2 minutes. The gas completely fills the principal

trunks almost instantly and apparently at a rather uniform rate. The lateral branches and spiracular connectives do not begin to fill until after the filling of the main trunks is complete, and they fill more slowly. Gas extends to the ends of the finest branches ("tracheoles"). Filling is initiated and progresses without any visible change in the normal locomotion and activity of the larva.

In normal larvae with spiracles in contact with air, careful and repeated observations under high magnification showed that gas first appears at some single point within the principal trunks, usually in one of the first four body segments. Although the suddenness of filling usually made it impossible to be absolutely certain of the exact spot where filling began, in a number of conclusive instances it began at some point other than a spiracle, and in no instance was it seen to begin at a spiracle. Ordinarily the gas spreads progressively and continuously both forward and backward from the starting point, and fills one main longitudinal trunk completely before crossing over by either the anterior or posterior commissures or both to spread through the tracheae of the opposite side. However, under conditions where filling has been interrupted experimentally (see below), it occasionally restarts in a new liquid-filled region, rather than continuing from its original stopping point.

Since the entire new system is still liquid-filled just after molting is completed, the tracheal liquid does not escape with the molted tracheae. Similarly, since the gas can be seen progressing distally into the liquid-filled branches (which end blindly), the liquid does not leave through any of the spiracles. This is particularly convincing after the first or second molt when the new system has only one pair of developed spiracles (anterior), and the gas can be seen passing posteriorly into regions where there are no possible exits. Furthermore, in larvae filling under oil (see below) no escaping aqueous liquid was seen. Also, liquid was sometimes seen to disappear from sctions of tracheae (usually the closed loops of the large lateral trunks) when gas approached simultaneously from both directions as Wigglesworth has also observed. From the above considerations it follows that the liquid passes through the tracheal and tracheolar walls into the blood and tissues as the gas appears.

Tracheal filling in submerged larvae

Since the open tracheal system of Sciara can, and probably normally does, fill independently of the spiracles, it was of interest to ascertain whether or not submerged larvae can fill their tracheae with gas. Of 27 late third instars submerged in a half inch of aerated water, 21 succeeded in molting in the course of 2 days, although they were not able to free themselves completely from the old cuticles for lack of a relatively dry surface for attachment. The tracheae of these larvae became gas-filled except for occasional individual tracheoles or segmental units. Normally no gas passes from the old to the new tracheae; but even if it did under the abnormal circumstances of the test, the amount would be far too small to fill the fourth instar system. As a possible indicator of whether the gas comes from an internal or external source, the experiment was repeated with freshly-molted larvae (i.e., with completely liquid-filled tracheae) taking care to exclude all air-bubbles from the dishes. Twenty or more larvae were used in each of three liquids: ordinary water,

freshly boiled water (the containers being filled to the top and covered), and a 6 mm. layer of mineral oil. In every instance filling began in approximately normal time and proceeded in the usual fashion. These experiments indicate that a visible external gas bubble is not necessary to initiate tracheal filling, in agreement with the previously described observations under high magnification. Furthermore, since the amounts of dissolved gas were probably quite different in the three liquids, the results suggest first that the gas which fills the tracheae of submerged larvae comes from some internal source, and second that filling is independent of O_2 tension over a wide range.

Tracheal filling in gases other than air

To investigate further the nature of the tracheal gas and the role of O_2 in filling, fresh ecdysiasts were exposed to commercial N_2, CO_2 and CO [1] in the gas chambers previously described.

Although there were considerable variations in the individual responses, the larvae were completely immobilized by a few minutes' exposure to any of the gases. In most instances gas appeared in portions of the main tracheae either in normal time or within 30 minutes and usually after the larvae were motionless. However, filling was not completed unless air was admitted to the chamber. Filling could be stopped and restarted repeatedly by alternating exposures to the tank gas and to air.

If the gases used were passed over hot copper gauze before admission to the gas chamber, filling was completely inhibited for an indefinite period (2 hours was the longest exposure tried). If the larva was returned directly to air after a period of complete anoxia not exceeding 75 minutes, filling usually was normal. Longer periods of complete anoxia often led to incomplete or delayed filling, and sometimes to complete and permanent inhibition. Permanently affected larvae died after a few hours, though they might, for a time, resume body movements, gut peristalsis and heartbeat. Filling was not visibly affected by pure medicinal O_2.

The quantitative relations between tracheal filling and O_2 tension, and differences between the specific effects of the individual gases, will be reported in detail in another communication, but for present purposes it may be said that, if given initially, mixtures of 0.3 per cent O_2 and 99.7 per cent CO_2, CO or N_2 suffice to permit gas to appear in the liquid-filled tracheae.

Effect of low temperature on tracheal filling

In freshly-molted larvae placed in small droplets of water on glass kept on melting ice, filling was indefinitely delayed (2¼ hours was the longest exposure tried). When returned to 25°, filling was normal but could be halted and restarted repeatedly by alternately chilling and rewarming the larva. During the exposure to 0° the larvae were practically motionless but responded markedly to mechanical stimulation.

[1] No difference was observed between experiments done in the light and those done in the dark, using a deep red filter to examine the larvae.

Experiments with hydrostatic and osmotic pressure

Attempts were made to prevent filling by exerting pressure from a coverglass on larvae mounted on a slide either in water or in air. It was found impossible to prevent filling from starting even with the greatest pressure which could be applied without bursting the larva.

The larval cuticle of Sciara is somewhat permeable to water, and exposure to hypertonic solutions causes withdrawal of water from the body. Since the consequent lowering of body turgor might accelerate filling, if release of hydrostatic pressure were a factor in filling, freshly molted larvae were immersed in double Ringer's solution. Filling was normal.

Discussion

From the foregoing experiments the following deductions can be made concerning some of the previous explanations of tracheal filling: (1) Tillyard's hypothesis necessitates a gradient between body and tracheal liquids of CO_2, a gas with high aqueous solubility and diffusion coefficient. This would be rather improbable even for larvae in air, but seems quite out of the question in Sciara larvae, in view of their demonstrated ability to fill in 99.7 per cent CO_2. (2) The fact that filling does not occur in larvae completely relaxed by anoxia argues against mechanisms involving a fall in hydrostatic pressure, as do the experiments with coverglass pressure and hypertonic solution. Conversely, the fact that filling can occur in larvae motionless in 99.7 per cent CO_2 runs counter to Wigglesworth's claim that CO_2-narcosis *per se* inhibits filling, and to Standtman-Averfeld's activity theory. Muscular activity as a factor also seems to be ruled out by the lack of filling after the body contractions induced in chilled Sciara larvae by prodding. (3) The inhibition of filling by anoxia argues against both osmotic pressure and imbibition as prime factors in filling, since both are claimed to be enhanced by anoxic catabolism. (4) Weismann and Keilin believed that the tracheoles were the site of absorption of tracheal liquid, but our findings that liquid can leave through trunk walls and that the trunks fill completely before gas enters any of the side branches indicate that the filling mechanism also operates through the main tracheae. This is supported also by the apparently rather uniform rate of filling in the main trunks (where a deceleration would be expected if the liquid were leaving via the side branches). An additional puzzle is that most reports agree that the largest trunks, which have the highest ratio of liquid content to surface area, fill in a matter of seconds; whereas, the fine, thin-walled branches, where it seems that imbibition, osmosis, etc. should be more effective, fill much more slowly. (5) The fact that the tracheal filling of the first instar does not occur for a day or more after hatching (if at all), whereas tissue osmotic pressure might be expected to be highest during the struggles of the embryo at hatching, militates against the osmotic theory. (6) From the fact that the Tenebrio embryo can fill its tracheae either with outside air or with gas derived from tissue fluids, Sikes and Wigglesworth concluded that there is no essential difference between filling in closed and in open systems. Although this conclusion does not appear justified logically, our finding that Sciara, which has an open tracheal system, normally may fill its tracheae with gas from some internal source, suggests that other insects (e.g., Tenebrio) likewise normally may fill as if they had closed systems in spite of opportunity to take in outside gas. (7) Our

demonstrations that (at ordinary temperatures) O_2 is an absolute prerequisite for tracheal filling, and that filling may be delayed by O_2-poor conditions, offer a possible explanation for the observations of Sikes and Wigglesworth that the Lucilia embryo will fill under water, but only if near the surface; of von Frankenberg that Corethra will not fill its tracheae if kept in boiled water; and of Tillyard that dragonfly larvae in O_2-poor water filled slowly. (The first observation was not elaborated by the authors; the second was attributed to insufficient dissolved gas in the surrounding water to serve for filling the vesicles; and the third to "weakening" of the larvae.) (8) The strict O_2-dependence of the initial filling of tracheae makes it questionable whether the "metabolic" movements of tracheolar liquid and gas studied by Bult are brought about by the same mechanism (though both processes are CO-insensitive) since Bult's postulated imbibitional mechanism is apparently anaerobic.

The net result of the above discussion is to eliminate from serious consideration as prime factors in tracheal filling all proposed mechanisms except those postulating "secretion" of gas. As previously stated, however, this concept is so vague that no critical consideration of it is possible at present. Insofar as "secretion" is a metabolic phenomenon, it is compatible with our finding that tracheal filling is absolutely dependent on O_2 and can be indefinitely inhibited by low temperature. However, we have no evidence as to what metabolic change might be involved, except that it is unlikely to be mediated by the cytochrome system. Numerous other aerobic processes (not all necessarily metabolic) are conceivable. Tillyard for example regarded O_2 as essential for a metabolic process by which gases dissolved in the external medium were transferred into the body and liberated in the tracheae.

It might, of course, be assumed that the *initiation* of filling and the actual filling process depend on quite different mechanisms. One could imagine for example that the reaction which requires O_2, and which might be metabolic, simply pulls a trigger which sets off a physical process resulting in tracheal filling. A number of possible mechanisms will be dealt with in another communication, but the evidence already available permits several deductions. Keister (1948) reported that scraps of air-filled third instar tracheae sometimes break off and are left within the new system at molting. The fact that filling will not start, or if previously started will not continue, in such larvae in the absence of oxygen or near O° C. shows that filling does not progress automatically once gas bubbles are present in the tracheal liquid. The further fact that filling, when it occurs, does not necessarily begin in regions containing tracheal scraps, shows that preformed gas bubbles are not a prerequisite for the initiation of filling. The same conclusions follow from the fact that after filling has been interrupted experimentally it sometimes resumes in a new (liquid-filled) region rather than continuing from its original stopping point. Finally, since filling will occur in gases ranging from tank O_2 to practically pure CO_2, CO or N_2 it is very unlikely that either the initiation or the progress of filling depends on the attainment of any critical ratio between gas concentrations, or upon the presence of any specific gas (except O_2).

Summary

(1) After each molt the new tracheal system of a Sciara larva in air normally remains filled with liquid for 3 to 8 minutes. It then fills spontaneously, rapidly

and completely with gas, beginning at some point in a main trunk. The gas comes from some internal source.

(2) Sciara larvae can molt under aerated water, and can fill their tracheal systems with gas while completely submerged in aerated or boiled water, or mineral oil.

(3) Tracheal filling can occur if the larva is in a mixture of 99.7 per cent CO_2, CO or N_2 with 0.3 per cent O_2, but not in complete absence of O_2.

(4) Tracheal filling is indefinitely inhibited near 0° C.

(5) Filling may be stopped and restarted repeatedly by alternating exposures to anoxic gas and air, or to low and room temperatures.

(6) Initiation and progress of tracheal filling are apparently independent of body movements, body hydrostatic pressure, and critical gas ratios.

(7) It is suggested tentatively that tracheal filling involves a metabolic process.

(8) A review and critique of previously proposed mechanisms of tracheal filling is presented.

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AN ABBREVIATED CONJUGATION PROCESS IN PARAMECIUM TRICHIUM

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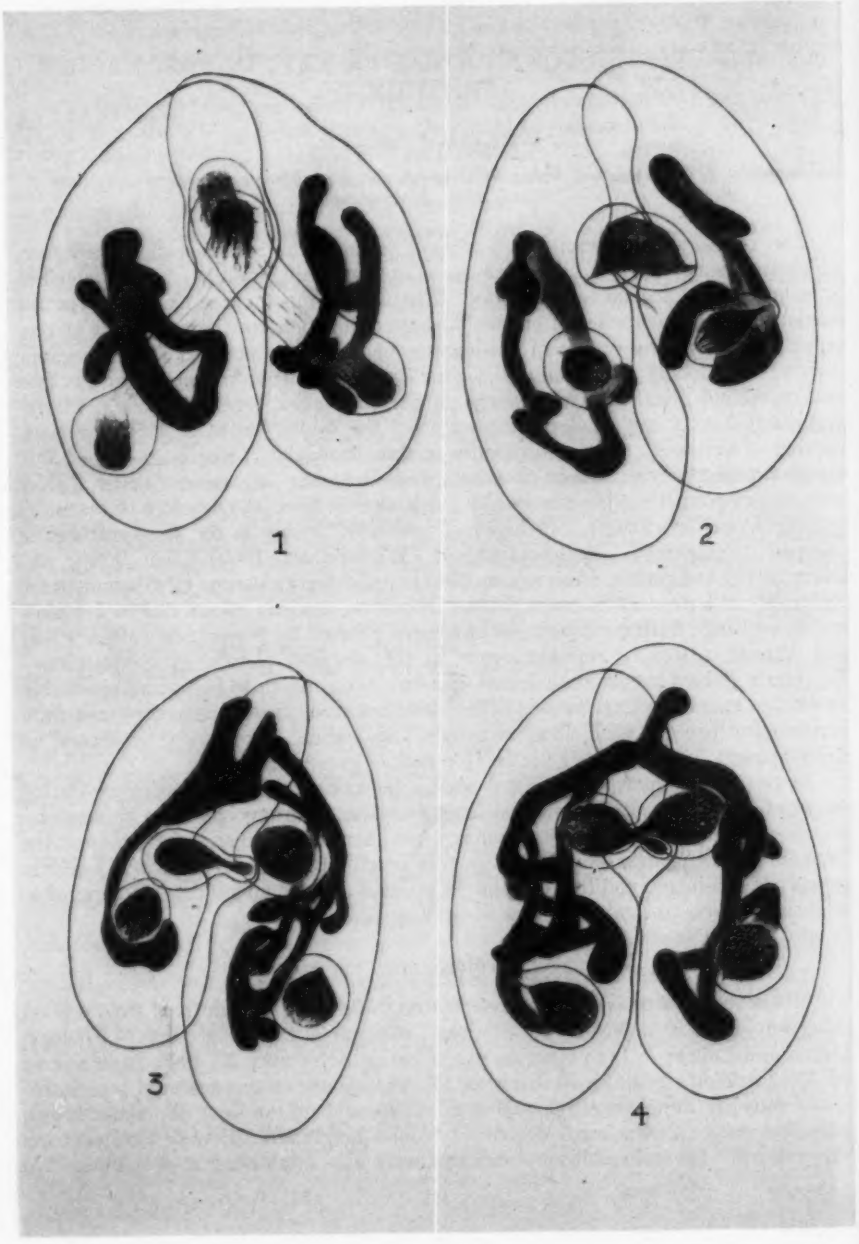
The remarkably constant, well-ordered, complex series of nuclear processes which are characteristic of ciliated protozoa during conjugation has been established by a host of cytological investigators. The almost monotonous regularity of the maneuvers in many species of ciliates during conjugation has led to a fairly stereotyped concept of the events of conjugation in ciliates generally; three pregamic divisions producing the pronuclei (with degeneration of nuclei after the first and/or second divisions), interchange of gametic nuclei, fertilization, and the reorganization of a new nuclear complex from the synkaryon after a characteristic number of divisions. The invariability of this "standard" process was called into question recently by a number of investigators including the author (Diller, 1936), who suggested that conjugation might not always involve an exchange of pronuclei and reciprocal fertilization, but fusion of pronuclei arising in the same member of the pair (autogamy). Both cytological (Wichterman, 1940; Chen, 1946; and Diller, 1948) and genetic (Sonneborn, 1947) studies have subsequently demonstrated the reality of autogamy in conjugation. Moreover, genetic effects due to cytoplasmic interchange during conjugation have been claimed by Sonneborn (1943, 1945) and Dippell (1948). Another event in the classical picture of conjugation—the puzzling third pregamic division—has now been shown to be not indispensable. In certain races of *P. trichium* (Diller, 1948) the conjugants may omit the third division and proceed with either reciprocal fertilization, autogamy ("cytogamy" of Wichterman) or parthenogenetic development of gametic nuclei.

In view of the great versatility of nuclear behavior shown by *P. trichium* during conjugation (Diller, 1948) and the large favorable micronuclei which this species possesses, it would seem to be of interest to describe a further variation from the "standard" conjugation behavior. In this heretofore undescribed process certain stages are eliminated and the micronuclei proceed directly and without degeneration of their products to establish a new nuclear apparatus.

Source of Material and Techniques

All the material on which the present study was made, was derived from a pond collection kindly furnished by Dr. Hannah Croasdale of the Department of Zoology, Dartmouth College. The collection was taken on September 20, 1946, from a pond on Dr. Carleton's grounds in Hanover, N. H. Some of this material was introduced into hay infusion on September 23, 1946, and on the next day about fifteen pure-line mass cultures, each descended from a single animal, were isolated from this culture. Several small mass cultures were also established at this time. No

PLATE I



significant differences in cytological behavior between the various lines were noticed, although most of them were examined from time to time and the selection of material to be studied was more or less random. Very shortly after their establishment, conjugation occurred in many of the cultures; for instance, in isolation culture No. 10, conjugation was in progress by October 3.

In most of the lines conjugation occurred in at least small numbers, at all times. The cultures were maintained on hay and malted milk, boiled in pond water. Several of them (isolation cultures Nos. 5 and 6) were mixed. Although there were small numbers of conjugants in each culture at the time of admixture, the combination resulted in a rather heavy incidence of conjugation so that it would seem as if these two cultures may have been opposite mating types. The cultures were maintained until June, 1947, when they were abandoned. Toward the end of their life span the cultures showed a more conventional behavior and finally did not conjugate at all. Temperatures in the laboratory became rather high and this may have been responsible for the decline of the cultures.

All the observations reported in this paper were made on killed and stained material. The animals were pipetted from the cultures into a centrifuge tube, concentrated, allowed to stand for a few minutes, and then fixed in Perenyi's fluid. They were sometimes subsequently treated with Schaudinn's fluid, and stained in acetic orcein or in Grenacher's alcoholic borax carmine. Both stains gave very good results. Usually fast green or indulin were used as counterstains. All the technique was carried out in the centrifuge tube and the animals mounted on slides in diaphane or clarite.

EXPLANATION OF PLATES

These are camera lucida drawings of stained whole animals from six isolation cultures started Sept. 24, 1946, and a small mass culture started Sept. 28, 1946, all derived from Carleton Pond, Hanover, New Hampshire. Magnification about 1200 times. All the figures illustrate *Paramecium trichium* during "abbreviated" conjugation. The animals were fixed in Perenyi's fluid and stained with Grenacher's alcoholic borax carmine or acetic orcein and counterstained with indulin or fast green. The specimens shown in Figures 1, 2 and 9 are representatives of small mass culture A; Figure 3, isolation No. 15; Figures 4 and 5, isolation No. 10; Figures 6, 7, 10, 11 and 16, isolation No. 14; Figure 8, isolation No. 6; Figures 12 and 15, isolation No. 9; Figures 13 and 14, isolation No. 17. Only the nuclear structures have been drawn. In some of the figures, particularly the later stages, old macronuclear fragments lying on top of the structures which were intended to be illustrated were omitted for the sake of clarity.

PLATE I

EXPLANATION OF FIGURES

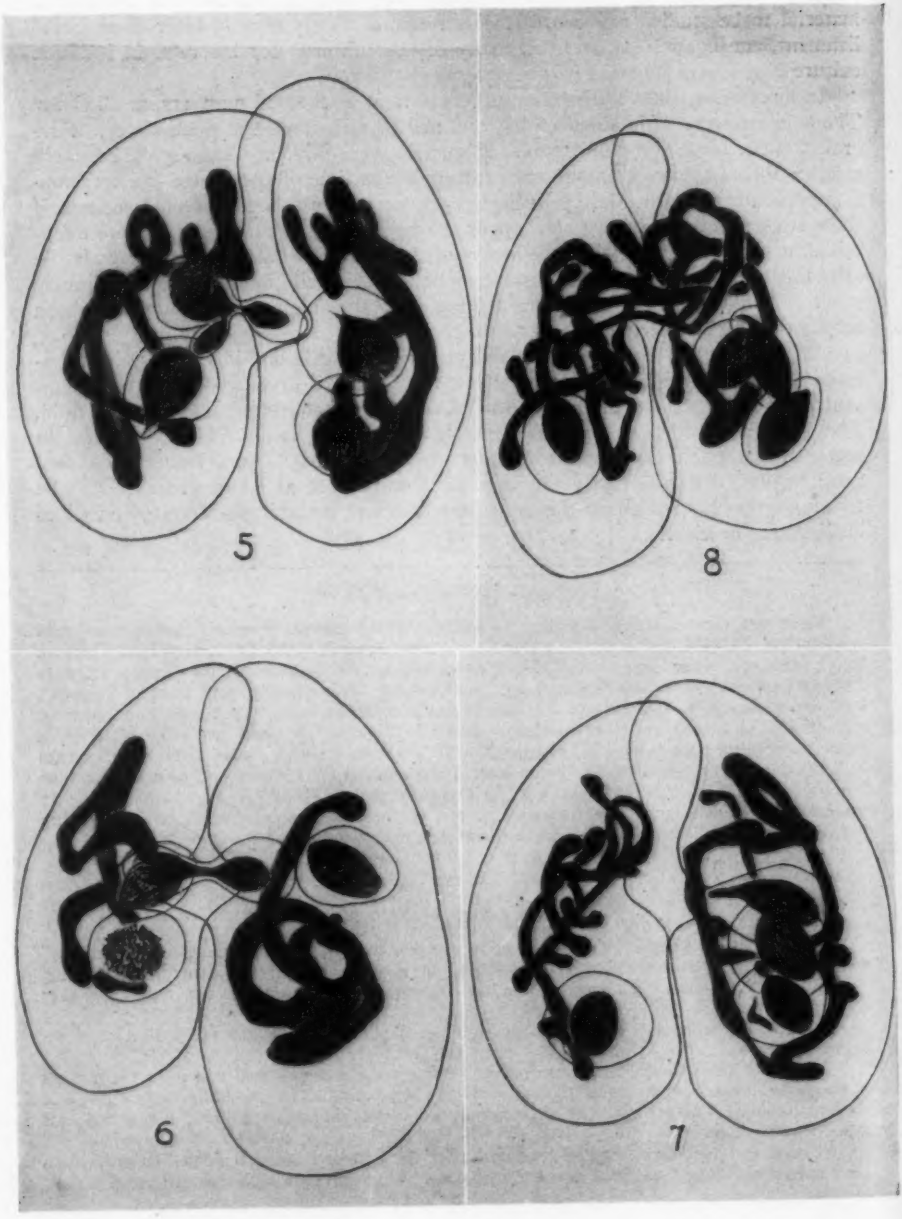
FIGURE 1. Telophase of first maturation division in a culture engaging in "abbreviated" conjugation. The macronuclear skeins (simple) seem to develop earlier than they do during "standard" conjugation. A daughter nucleus is found in the paroral cone region of each conjugant.

FIGURE 2. Two nuclei resulting from the first division. Each has a tail indicative of recent separation.

FIGURE 3. Two nuclei in each conjugant. Twisted chromosomes and a knob on the nucleus in the paroral cone region of the left conjugant are suggestive of its impending passage. Since the corresponding nucleus in the right conjugant is not in a similar condition, this pair suggests a one-way passage.

FIGURE. 4. "Migratory" nuclei produced after the first division. A nipple-like process on each extending toward external boundary of cone. Suggestive of reciprocal transfer.

PLATE II



Observations

Although no observations were made on the length of time that the members of the pair remain attached in abbreviated conjugation, it is my distinct impression that the time is much less than for the conventional method. It is probable that the first division of the micronucleus consumes less time than ordinarily is the case. The macronucleus in abbreviated conjugation seems to be somewhat precocious in its skein formation. By the time of the telophase of the first division, a simple macronuclear skein (Fig. 1) has formed. One sister chromosome group of each spindle is likely to be found in, or near, the paroral cone. The daughter nuclei, immediately after separation, frequently have "tails" on them (Fig. 2). This is true also for the corresponding post-telophase stage of the other divisions. Normally, the first division is not followed by degeneration. I have seen only one or two pairs, in abbreviated conjugation, in which degeneration of nuclei was evident. This is rather unusual because in conventional conjugation, as well as in the type in which only the third pregamic division is omitted, degenerating nuclei after the first and second divisions are the rule.

A reorganization of the two nuclei in each conjugant leads to a premetaphase condition (Fig. 3 and others). A knob-like, nipple-like, or handle-like process (Figs. 3, 4, 5, 6, 7, 8 and 10) is formed on one (Fig. 3) or two (Fig. 4) of the nuclei. This modification marks the nucleus as a potential migratory gametic nucleus. Undoubtedly, it is reflective of cytoplasmic stresses, pressures and/or currents in the cone regions. The narrow pointed process may be directed toward the cell boundary or toward the interior of the cell. The chromosome threads are frequently arranged in a spiral fashion, suggesting a twisting influence on the nucleus. In Figure 3, the presence of a single nucleus in the paroral cone of the left conjugant, with a terminal knob, and the absence of a similar structure in the right conjugant suggest an imminent one-way passage. Frequently, the macronucleus of one conjugant is a little more advanced in skein formation than is the other (Fig. 3). In contrast, two such migratory nuclei (Fig. 4) may be present, indicating an approaching reciprocal transfer.

Occasionally the pinching effect appears to be so severe as to cause a disruption of the migratory nucleus into several parts. Such an instance is represented in Figure 5. It is conceivable that this process may be the means whereby small accessory nuclei arise, by a purely amitotic mechanism. This possibility will be considered later in connection with subsequent stages. It is probable that most of these constricted nuclei would recondense and adjust to the normal condition after

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Plate II

Explanation of Figures

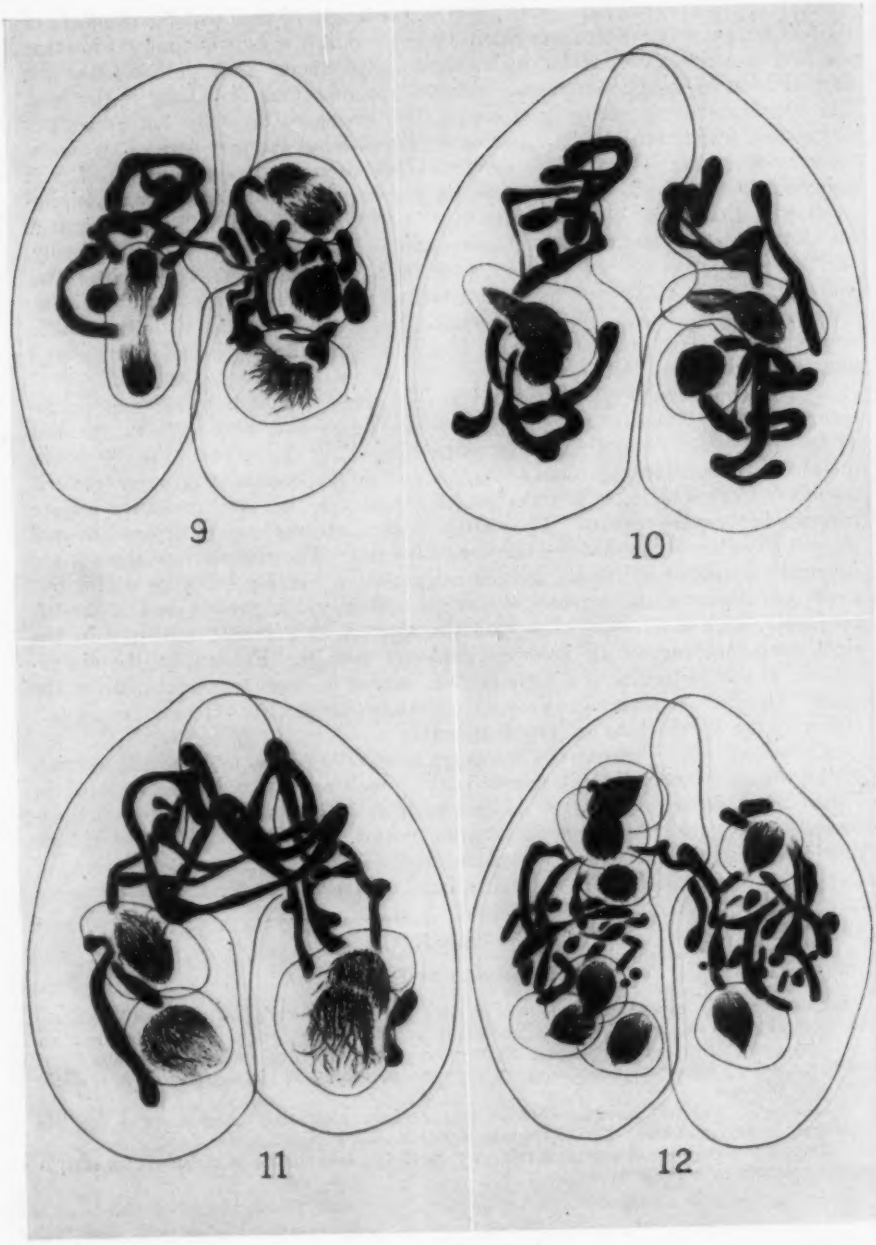
Figure 5. The "migratory" nucleus of the left conjugant pinching off two small portions, each connected to the larger section. One of the small accessory nuclei is lying in the cone.

Figure 6. Macronuclei in simple skeins. No degenerating micronuclei. Migratory nucleus, constricted in the middle, passing through the cone from the right conjugant to the left. One-way passage.

Figure 7. One-way passage of migratory nucleus, after first division, from the left conjugant to the right one. Tail of migratory nucleus still in tip of cone.

Figure 8. One-way passage of a migratory nucleus. Interchange of rather coarse macronuclear strands in both directions.

PLATE III



the temporary stresses had been relieved. I have seen one other instance, not figured in this paper, which could produce a similar result. One member of the pair had two normal nuclei from the first division. The other had a tripolar telophase of the first division. One of the three sister chromosome groups was smaller than the other two.

At the time when the migratory nuclei are actually passing through the paroral cones they often show an equatorial attenuation. Such a dumbbell effect is shown in Figure 6. The migratory nucleus is passing into the small left conjugant. There is no indication of nuclear passage in the reverse direction. The macronuclear skeins are still relatively simple and coarse. In this case there is cytoplasmic continuity at one level only. Figure 7 illustrates a slightly later stage of one-way passage of a migratory nucleus after the first division. Its tail is still in the cone region. Probably in this pair there is cytoplasmic continuity between the conjugants at two levels.

It is difficult to ascertain the frequency of the occurrence of the different modes of behavior of the nuclei, after the first division, in the Carleton Pond stock of *P. trichium*. One-way passage of a nucleus, leading to the spectacular unbalanced condition of three nuclei in one member and one nucleus in the other conjugant—a situation which first attracted the author's attention to this process—is by no means a rarity in these stocks. Two-way passage (interchange), as suggested by Figures 4, 10 and others, is also quite common. In the event of an original heteroploidy of the micronuclei of the two conjugants, it is possible to determine at a later stage whether interchange had occurred. A third alternative is evident: the failure of nuclei in both conjugants to migrate and their development in the same conjugant in which they arose. This possibility is, in the author's opinion, a valid one, but seems to be more rare than the other two. Apparently, breakdown of the tips of the paroral cones, cytoplasmic currents, and/or internal pressures at the proper stage are the factors which determine the movements of nuclei at this time. The nature of these forces is entirely conjectural but it is of interest to note that they may be unequal in the two members of the pair.

Shortly after the passage of the micronuclei, or their non-passage, strands of the macronuclear skein may, or may not, become stretched across the cone regions from one conjugant to the other in much the same fashion as in unabbreviated conjugation (Diller, 1948). Passage of the macronuclear skein may be unidirectional

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PLATE III

EXPLANATION OF FIGURES

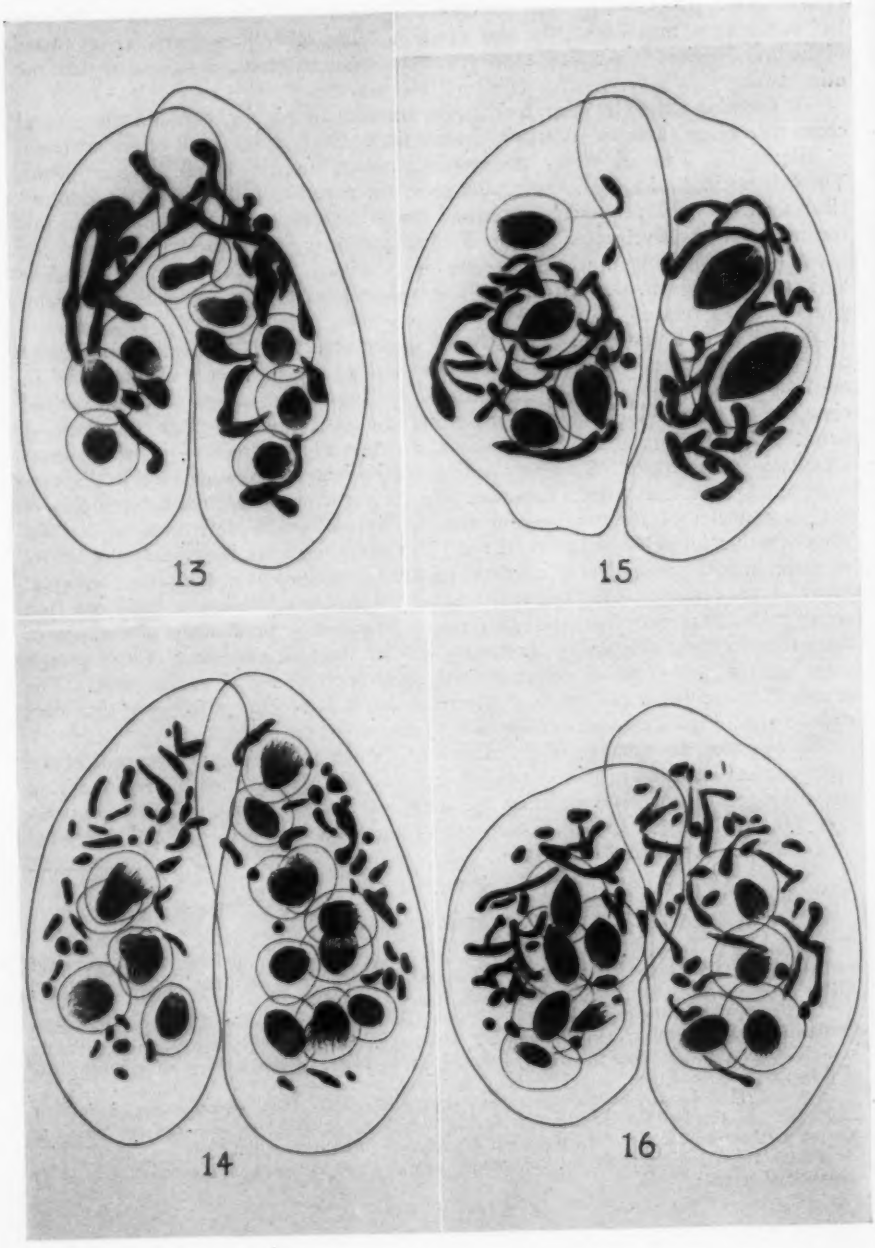
FIGURE 9. One micronuclear figure in left conjugant; three dividing nuclei in right conjugant. Second division. Macronuclear strand passing from left conjugant into right. All the nuclei are in slightly different mitotic stages.

FIGURE 10. Two gametic nuclei in each conjugant. Either cross-fertilization, autogamous fertilization, or parthenogenesis, is imminent. Uncertain whether there is cytoplasmic continuity in oral cone regions. The "migratory" nuclei are retaining the constrictions characteristic of the migratory condition.

FIGURE 11. Synkaryon formation in right conjugant. Two separate nuclei in left conjugant. In the latter, either nuclear fusion is delayed or the gametic nuclei are going to develop parthenogenetically. Macronuclear interchange in both directions.

FIGURE 12. Six nuclei in left conjugant; two in right conjugant. Macronuclei in short complicated strands.

PLATE IV



(Fig. 9) or may extend in both directions (Figs. 8 and 11). Macronuclear exchange was very frequent in the Carleton stock. In case it is unidirectional, the strands can pass either from the uninucleate conjugant into the trinucleate member, as in Figure 9, along the path which the single migratory nucleus took, or in the reverse direction. The direction of macronuclear movement seems not to be directly correlated with the direction of micronuclear movement. Exchange of macronuclear material apparently marks the end of interconjugant micronuclear movement.

As remarked above, there is normally no degeneration of nuclei at this stage, or any other stage, in abbreviated conjugation. One can detect several alternative modes of behavior of the nuclei from this point, keeping in mind the possibilities that the nuclei of one member of the pair may be behaving differently from those of the other member, and even that the nuclei in the same conjugant may be diverse in their activities. First, fertilization (synkaryon formation) may occur. Such a condition is shown in the right conjugant of Figure 11 and such was probably the ancestry of the two nuclei in the right member of Figure 15. Depending on whether interchange had occurred, cross-fertilization or self-fertilization would be accomplished. Second, parthenogenetic development of the nuclei may take place. This seems to be the most frequent type of activity in abbreviated conjugation. Third, combinations of fertilization and parthogenetic development may be adopted. Although the critical stages are rare, it is possible by reason of size differences to reconstruct previous history.

Figure 9 illustrates the micronuclear activity of the second division. The conjugant on the left contains a late anaphase micronucleus, while that on the right has three dividing nuclei in slightly different mitotic stages. It is a little unusual for the micronuclei to show such asynchrony. Probably the two anaphase nuclei, one in each conjugant, are sisters. It seems likely that all of these nuclei are developing without fertilization (parthenogenetically).

It is difficult to be sure about the exact history and the immediate fate of the nuclei of Figure 10. The "tailed" nuclei may have been interchanged, or not, and may be on the point of fusion with the stationary nuclei. Otherwise, parthenogenetic development would be expected to follow.

Figure 11 shows synkaryon formation in the right conjugant and two separate

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PLATE IV

EXPLANATION OF FIGURES

FIGURE 13. Unusual interchange at the end of the second division, with no degenerating nuclei apparent. The nucleus at the top is pressing against the cone moving toward the left, while the nucleus directly below it is part-way through the cone, passing into the right conjugant.

FIGURE 14. Six nuclei in left conjugant. Ten nuclei in right conjugant. Presumably, this condition arose from a one-way passage of a gametic nucleus at the stage represented in Figure 13. Old macronuclei represented by closely packed short rods and spheres (many omitted).

FIGURE 15. Four nuclei in left conjugant. Two very large nuclei in right conjugant. Probably parthenogenesis has occurred in the left conjugant while synkaryon formation has taken place in the right member.

FIGURE 16. Four nuclei in right conjugant. Four large nuclei and two small ones in the left conjugant. The latter may have arisen from pinched-off parts of nuclei after the first division, as suggested by Figures 3 and 5. (They may have originated in the right conjugant).

nuclei in the left conjugant. In the latter, either nuclear fusion is delayed, or the gametic nuclei are going to develop parthenogenetically. The latter alternative seems to me the more probable, since my observations suggest that very little time elapses before nuclear fusion is completed.

Occasionally I have found that one or both of the conjugants at later stages possess nuclei of different sizes. Aside from the explanation of original heteroploidy of the conjugants, this can best be interpreted by assuming that the larger nuclei have arisen from synkarya, while the smaller ones have developed parthenogenetically. Assuming that the number of nuclear generations is the same in both conjugants, synkaryon formation in one and not the other will result in different numbers of nuclear products in the two members at later stages (cf. Fig. 15). Although this is not the only explanation, I believe that asymmetric synkaryon formation is a valid one. One wonders whether an extra postzygotic division is required for final reorganization since there has been a reduction in nuclear number in the conjugant which produced a synkaryon. Another device for bringing about unequal nuclear numbers in the two conjugants is for the mitotic stages to become slightly out of step with each other. I believe this to be a real, but rather rare, happening. However, in the uninucleate-trinucleate pairs the single nucleus seems often to be ahead of the three others (Fig. 9).

Figure 12 shows completion of the second division in a pair in which there has been one-way transfer. By this time the macronucleus has usually fragmented into complicated short strands and rodlets and no longer can be traced to the opposite cell. Apparently the paroral cone intercommunications heal over at this time. The conjugants separate after this stage or during the next (third) division.

Migration of nuclei in abbreviated conjugation is not completely restricted to the time immediately after the first division. Very rarely, interchange can occur after the second division. Two such cases are shown in Figures 13 and 14. In the former, each conjugant has four nuclei, one of which is located in the paroral cone and is projecting into the other animal, apparently on the verge of effecting interchange. A one-way transfer of this type would result in five nuclei in one conjugant and three in the other. At the conclusion of the third division of such a hypothetical case, six and ten nuclei, respectively, would be found in the conjugants. That is apparently the explanation of the asymmetric condition of the pair illustrated in Figure 14.

A rather unusual and interesting asymmetrical case is illustrated in Figure 16. Four nuclei, following the second division, are present in each conjugant. In addition, there are two small nuclei in the left conjugant. These may have arisen as "buds" pinched off the nucleus at the time of the first division, as suggested earlier, which have persisted through division. (See Fig. 5.) They seem perfectly viable and normal.

The events subsequent to the third division, when the animals separate, have offered no special points of interest. Presumably reorganization, anlagen formation, and disappearance of the old macronucleus are similar to the standard processes characteristic of *P. trichium* (Diller, 1948), although I have made no particular effort in these studies to work out the post-conjugant stages. Regularly, the mature exconjugants would be expected to have four macronuclear and one micronuclear anlagen.

Discussion

The abbreviated conjugation process in the Carleton race of *P. trichium,* reported in this paper, accomplishes the ends of nuclear reorganization in a remarkably simple and direct manner without wastage of micronuclei and without unnecessary stages. However, it is so unorthodox and so divergent from the conjugation pattern of other ciliates, and even of other races of the same species, as to pose problems about its general significance, and, in fact, about the meaning of certain phases of the conjugation process as a whole. The standard or conventional scheme of micronuclear activity in the ciliates involves three pregamic divisions (two have been reported in a certain race of *P. trichium,* Diller, 1948), and a variable number of postzygotic divisions which reconstitute the definitive nuclear complex. The term "postzygotic divisions" is here extended to include parthenogenetic divisions or generations as well as those of fertilization nuclei. It is borne in mind, of course, that variation in numbers of macronuclear and micronuclear anlagen is common but is fairly constant for a given species. In *P. trichium,* in the standard process, there are two or three pregamic divisions and three postzygotic divisions. In abbreviated conjugation three divisions, simply, are required to complete the process. (Possibly an extra division is appended in case synkaryon formation is involved.) Similar numbers of final nuclear products arise in both processes. The failure of nuclei to degenerate in the abbreviated process, generally, accounts for the end products being the same in number as in standard conjugation. The question then arises as to the homology of the nuclear generations in abbreviated conjugation with those in the standard process. In both, the first division shows a characteristic polarized (not crescentic) prophase stage. This may well be indicative of a maturation or a reductional process and is followed, in the standard conjugation, by one or two other divisions before fertilization or parthenogenetic development. However, in abbreviated conjugation there is no further division before nuclear exchange and fertilization (or parthenogenesis) are accomplished. If one were to assign the exchange period as a central reference point in both processes, then one can consider the first division in abbreviated conjugation as a maturation division and the second and third divisions as being homologous with the postzygotic divisions of the standard process. If this interpretation is valid, what can be inferred about the chromosomal cycle in abbreviated conjugation? A comparable problem was raised before (Diller, 1948) in connection with the omission of the third division in certain races of *P. trichium* and the parthenogenetic development of reduced nuclei; it was concluded that under these circumstances each conjugation would be expected to result in a progressive diminution of chromosome number. Unfortunately, direct observation of chromosome numbers in the various generations is very difficult, if not impossible, to make, and even estimates of nuclear size are not very satisfactory in spite of the large and comparatively favorable micronuclei of *P. trichium.* It has been considered axiomatic that two maturation divisions are necessary to bring about chromosomal segregation and reduction in mature gametes. This is undeniably accomplished in the standard conjugation process, even when the third pregamic division is omitted, but is doubtful in abbreviated conjugation.

Two possibilities suggest themselves. First, that reduction is completed in the later divisions and that the final nuclei are haploid, unless fertilization occurs. In

the latter eventuality, the awkward situation of the occurrence of a maturation division before fertilization and another after fertilization would exist. A second, and more probable, speculation is that the gametic nuclei are not reduced but diploid, and the nuclei arising by parthenogenesis would remain diploid while those derived from synkaryon formation would be tetraploid (cf. Fig. 15). Although a good deal of heteroploidy was evident in these cultures, hypoploidy was not nearly as extreme nor as conspicuous as in certain other stocks which I have been studying.

In correlation with the shortened morphological manifestations, it would be interesting to know how the time relationships of abbreviated conjugation compare with those of the standard process. I have the impression that abbreviated conjugation takes a shorter time than the standard process, but no positive evidence on this point. Unfortunately, the cultures were discarded before this information was obtained, in fact, before it was realized that abbreviated conjugation was happening; and I have not been able to secure any more stocks from the Carleton Pond, although several collections were made. The causes of the induction of abbreviated vs. standard conjugation are also entirely unknown at present. It seems to be not entirely a racial or genetic effect, since there were some instances of standard conjugation in certain of the Carleton Pond stocks.

I know of no other conjugation study in ciliates in which nuclear transfer has been observed at the end of the first division. The mechanism of conjugation activity has apparently been accelerated to bring about nuclear passage two generations ahead of the time usually required: the tips of the paroral cones have broken down and the macronuclear skein is far advanced. The latter seems to be precocious and attuned to the prospective activity of the micronuclei. Transfer of the micronuclei may be unidirectional, resulting in the asymmetrical condition of one nucleus in one member and three in the recipient, or reciprocal (interchange), or, probably, there can be non-passage. Such a selection suggests a chance determination. A pinching or constriction of the "migratory" nucleus before and during passage may be extreme —so severe as to cause a complete separation of fragments from the nuclei. These may persist and continue an apparently independent existence. (One hesitates to apply the terms "migratory" and "stationary" nuclei to the products of the first division, with the implication that these are reduced nuclei and that they invariably are involved in interchange.)

As in other accounts of conjugation in *P. trichium* (Diller, 1948), macronuclear passage may occur in abbreviated conjugation, after micronuclear migration. The macronuclear exchange may be either reciprocal, unidirectional, or, probably, omitted. In case of unidirectional micronuclear passage, macronuclear exchange is not necessarily along the same path, i.e., from the conjugant with one nucleus into the one with three nuclei, but may be in the opposite direction from the trinucleate to the uninucleate conjugant.

Also, as in other processes of conjugation in *P. trichium*, the subsequent micronuclear activities may be variable: fertilization (cross-fertilization or autogamy), parthenogenetic development, or combinations of fertilization and parthenogenesis in the two members of a pair or even, probably, in the same member of the pair. The versatility, lability and variability of micronuclear activity in *P. trichium* should be susceptible to experimental attack and analysis.

SUMMARY

1. A process of "abbreviated" conjugation occurs in one race of *P. trichium* in which the number of micronuclear divisions is reduced to three (or possibly four) from the "standard" pattern of five or six.

2. There may be exchange of micronuclei at the conclusion of the first division. Frequently, unidirectional passage of a gametic nucleus occurs at this time so that an asymmetry results in the two conjugants, one of them having three micronuclei and the other conjugant one micronucleus.

3. The products of the first division proceed, directly, to reconstitute the new nuclear apparatus. This they do by synkaryon formation, parthenogenetic development or a combination of the two, usually dividing twice. There is no degeneration of nuclei between divisions.

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ADDENDA

Seminar, July 12, 1949, Marine Biological Laboratories

X-ray mutations and fecundity of Mormoniella. Marion E. Kayhart and P. W. Whiting.

Females were treated (4000–8000 r) and mated to untreated males. Lowest dose given is, by analogy with Habrobracon, well above lethal for metaphase eggs. Therefore all offspring must be produced from prophase or preprophase. Fecundity tests show decrease in offspring with increasing dose given to adult females—(1173/10) 117.30 per untreated female, (229/27) 8.48 per 4000 r treated, (91/40) 2.27 per 5000 r, (199/88) 2.26 per 6000 r, (105/86) 1.22 per 7000 r, (69/83) 0.83 per 8000 r. Treatment (4000 r) of middle-aged pupae is more effective—(26/40) 0.65 offspring per female—than of young pupae—(113/50) 2.26—, old pupae—(445/80) 5.56—, or adults. Treatment (6000 r) of middle-aged pupae is more effective—(36/40) 0.90 per female—than of old—(88/40) 2.20—or adults. This sensitivity of eggs of middle-aged pupae is probably due to injury to nurse and follicle cells, as well as to oocytes.

Among 1262 sons of treated mothers were five independently occurring eye color "mutants" —scarlet-2 (found dead), scarlet-3 (overetherized), pinkish (sterile), oyster (proved *oy*), tomato, *to* (fertile). Among 387 daughters given breeding test, in general producing over 30 sons each, 16 proved heterozygous for mutant traits—eye colors: oyster, *oy* (fertile), scarlet, *st* (fertile), vermilion, *vm* (fertile) (4 + : 5 mutants), vermilion (proved *vm*), garnet, *ga* (fertile) (58 + : 6 mutants), scarlet-4 (fertile) (14 + : 19 mutants, scarlet-5 (fertile) (50 + : 58 mutants), light scarlet (28 + : 9 mutants found dead); body colors: blue-1 (19 + : 29 mutants found dead), blue-2 (sterile) (1 + : 3 mutants), blue-black (sterile) (124 + : 57 mutants), greenish-blue (sterile) (33 + : 23 mutants), purple (sterile) (15 + : 12 mutants); wings: shredded (sterile) (37 + : 20 mutants); legs: short-1 (sterile) and short-2 (sterile). No mutations have been found among many thousands examined from untreated stock. The 22 from the treated were distributed irrespective of dose or of age at time of treatment.

Seminar, August 16, 1949, Marine Biological Laboratories

The Development of Menidia-fundulus Hybrids. James M. Moulton.

Menidia beryllina beryllina ♂ × *Fundulus heteroclitus* ♀ hybrids survive until the hatching of controls, as reported and briefly described by Clark and Moulton (*Copeia*, 1949, No. 2), but have thus far failed to hatch. The reciprocal cross advances only to the yolk-plug stage. Both crosses of the other *Menidia* race found in the Woods Hole region, *Menidia menidia notata*, with *Fundulus heteroclitus* survive only to the yolk-plug as shown by Moenkhaus (1904, *Am. J. Anat.*, 26–65). The possibility is suggested that *M. b. beryllina* may have been the form used by J. Loeb in his hybridization experiments (1912, *J. Morph.*, **23**, 1–15; 1915, *Biol. Bull.*, **29**, 50–67).

Naming the hybrid embryo according to the egg used, the *M. b. beryllina* hybrid begins to lag in its developmental rate as compared with that of controls by the fifth cleavage—about three and a quarter hours at 18.5 degrees C. The *Fundulus* hybrid shows the first signs of such a lag at about 16 hours, when the expanding blastula is present in the controls. Subsequent to these stages it is rather difficult to distinguish between the effects of developmental lag and the origin of other anomalies in producing differences between hybrids and controls.

Among the anomalies observed in the hybrids are failure in yolk resorption which may be associated with other irregularities, incomplete development of the circulatory system and a rather amorphous nature to the embryo itself. Common anomalies involve the eyes. Some batches of hybrids have demonstrated such anomalies in nearly 100% of the embryos, the defects ranging from an approach of the optical organs to each other, through a perfect cyclopian condition, to a complete absence of optical structures so far as visible externally.

ERRATA

THE BIOLOGICAL BULLETIN, VOLUME 97, No. 2, P. 260

Extrusion of jelly by eggs of Nereis limbata under electrical stimulus. W. J. V. OSTERHOUT.

In the first paragraph of the abstract noted above, "17 milliamperes" should read "170 milliamperes" and "much larger direct currents" should read "comparable direct currents."

In the third paragraph, "17 milliamperes" should read "170 milliamperes."

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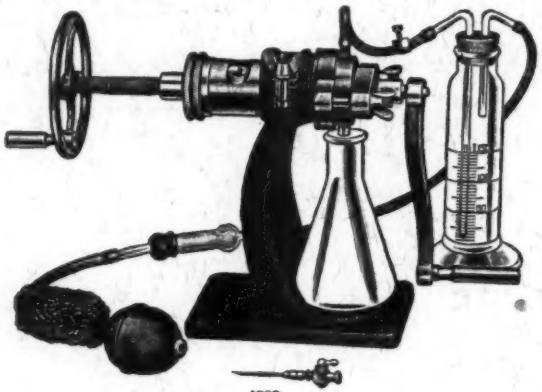

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